MCH precursor-derived peptides in tilapia

Identification, gene expression & function

Diet Gröneveld

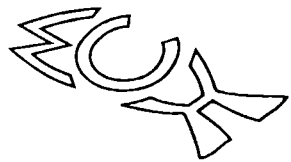
MCH precursor-derived peptides in tilapia

Identification, gene expression & function

Diet Gröneveld

MCH precursor-derived peptides in tilapia

Identification, gene expression & function



een wetenschappelijke proeve
op het gebied van de Natuurwetenschappen

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Katholieke Universiteit Nijmegen,
volgens besluit van het College van Decanen
in het openbaar te verdedigen
op woensdag 4 oktober 1995
des namiddags te 3.30 uur precies

door

DIANE GRÖNEVELD
geboren op 11 juli 1966 te Enschede

Ponsen & Looijen, Wageningen

Promotor: Prof. Dr. S.E. Wendelaar Bonga

Co-promotor: Dr. P.H.M. Balm

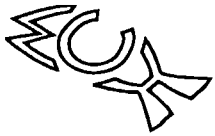
Dit proefschrift kwam tot stand met financiele steun van de Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO) via de Stichting voor Levenswetenschappen (SLW).

aan mijn ouders

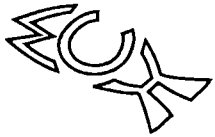
CIP-GEGEVENS KONINKLIJKE BIBLIOTHEEK, DEN HAAG

Groneveld, Diane

MCH precursor-derived peptides in tilapia
identification, gene expression & function /
Diane Groneveld - [S l s n] - Ill
Proefschrift Katholieke Universiteit Nijmegen -
Met lit opg
ISBN 90-9008456-8
Trefw neuro-endocrinologie / tilapia



Contents



Chapter 1

General introduction

SCOPE OF THE THESIS

Living organisms adapt to changes in their environment in order to survive. Animals possess three basic systems that control the regulatory events involved in these adaptations: the nervous, the endocrine and the immune system.

The nervous system consists of neurons, which are especially equipped for fast responses to environmental challenges by quick transfer of messages. A neuron receives input from sensory organs or other neurons, translates this input into an electrical signal, the action potential, which is sent along the axon to a target. The communication between neurons and their targets (including other neurons) occurs mainly by synaptic transmission. A synapse contains a presynaptic (axonal) and a postsynaptic (target) membrane and an intersynaptic space, the synaptic cleft. An arriving action potential can induce the release of a chemical messenger, such as a neurotransmitter or a neuropeptide, from the presynaptic site into the synaptic cleft. The neurotransmitters or -peptides affect the target cell by binding to postsynaptic receptors or ion channels [Kandel *et al.*, 1991].

The endocrine system consists of secretory cells that release their messenger molecules, (glyco)peptide or steroid hormones, into the circulation. In contrast with synaptic transmission, characteristically there is a long distance between endocrine cells and their targets, which results in a slower communication process. Synthesis and release of hormones is controlled by the nervous system, or by chemical messengers from other endocrine organs. The endocrine system is in particular involved in the control of long-term physiological processes, such as growth, reproduction and adaptation [Norris, 1980].

The immune system consists of a variety of cells, producing many intercellular messenger molecules (e.g. cytokines and antibodies). This system primarily acts against infectious agents [Roitt *et al*, 1989], while recently interactions with the nervous and endocrine systems have been described [e g. Blalock, 1989]. The neural and the endocrine systems also communicate via neuro-endocrine mechanisms. This thesis aims to gain more insight in the peptidergic communication between the nervous and the endocrine system during adaptation processes in fish. For this purpose a multidisciplinary study has been carried out on the

interactions between hypothalamic neurons producing the novel heptadecapeptide melanin-concentrating hormone (MCH), and endocrine pituitary cells of the cichlid teleost tilapia (*Oreochromis mossambicus*) In particular, the actions of MCH neurons with respect to control of background adaptation, stress response and ion balance have been investigated

THE HYPOTHALAMUS AND PITUITARY GLAND OF TELEOST FISH

The hypothalamus-pituitary axis forms a major center of neuro endocrine communication As in all vertebrates, the pituitary gland of teleosts can be divided in a neurohypophysis and an adenohypophysis Many axons originating from hypothalamic nuclei terminate in the neurohypophysis These axons transport, store and release neuropeptides and hormones In the adenohypophysis endocrine cells are located The structural organization of the teleost pituitary gland is very different from that of other vertebrates The main differences are the absence of - or an at most poorly developed - median eminence and the deep penetration of the neurohypophysis into the adenohypophysis with direct nervous connections between neurohypophysis and adenohypophysis (Fig 1 1)

On a sagittal section, three distinct areas in the adenohypophysis are easily discerned (Fig 1 1) the rostral pars distalis (RPD) is located most anteriorly, it is also called anterior lobe (AL) This region contains prolactin (PRL) and adrenocorticotropin (ACTH) cells Most posteriorly the pars intermedia (PI) is located, which contains cells producing α-melanocyte-stimulating hormone (α-MSH) and somatolactin (SL) A region called proximal pars distalis (PPD) is located in between the RPD and PI, and contains growth hormone (GH), gonadotropin (GTH) and thyroid-stimulating hormone (TSH) cells The PI together with the neurohypophysis is named neuro-intermediate lobe (NIL)

Peptides released from the neurohypophysis can either act as hormones, that are released into the general circulation, or as neuropeptides that regulate the secretory activity of pituitary endocrine cells, or in both ways Examples of neurohypophysial hormones are arginine vasotocin and isotocin, both belonging to the vasopressin family [Perks, 1987], and MCH [Baker, 1991] Neuropeptides of the neurohypophysis regulating adenohypophysial cells include growth hormone-releasing factor (GRF), corticotropin-releasing hormone (CRH), thyrotropin-releasing hormone (TRH), gonadotropin-releasing hormones (GnRHs), enkephalin, glucagon like peptide, FMRFamide-related peptides, somatostatins (somatostatin-14), galanin, neuropeptide Y (NPY), bombesin, vasointestinal peptide (VIP), gastrin-like peptides [Batten *et al*, 1990, Peter *et al*, 1990], and also MCH [Barber *et al*, 1987] Many are produced in

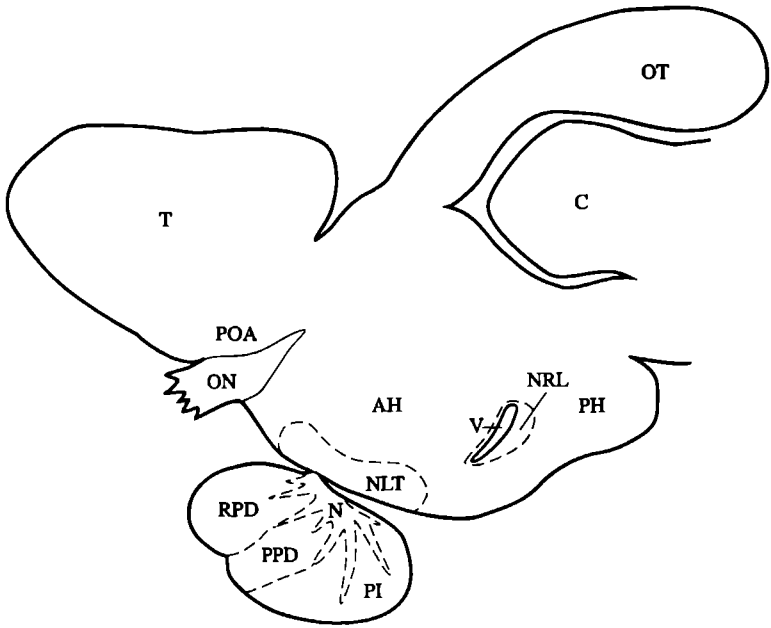


Figure 1.1 Representation of a near midsagittal section of the forebrain and pituitary gland of a teleost fish. Abbreviations: AH, anterior hypothalamus; C, cerebellum; N, neurohypophysis; NLT, nucleus lateralis tuberis; NRL nucleus recessus lateralis; ON, optic nerve, OT optic tectum; PH, posterior hypothalamus; PI, pars intermedia; POA, preoptic area; PPD, proximal pars distalis; RPD, rostral pars distalis; T, telencephalon; V, lateral ventricle. [Adapted from Olivereau, 1967, Doerr-Scott, 1976, Peter & Gill, 1990].

the preoptic nuclei of the hypothalamus, but several other hypothalamic nuclei are involved as well.

BACKGROUND ADAPTATION IN TELEOSTS

A well known model to study the peptidergic communication between neural and endocrine cells is the process of background adaptation. Lower vertebrates (e.g. some teleosts, amphibians and reptiles) are capable to alter the colour of their skin in response to background colouration. This colour change permits the animals to modify their skin colour to match the background, rendering it less visible to predators or prey [Baker, 1993].

Changes in skin colour are usually the result of pigment migration in skin melanophores When the pigment melanin is aggregated around the nuclei of the melanophores the skin appears pale, whereas melanin dispersion leads to a dark skin colour In teleosts the regulation of skin pigmentation occurs in two ways, by the nervous system and by hormonal control The skin is innervated by fibers of the autonomic nervous system containing the neurotransmitters noradrenalin and/or acetylcholine [Fujii & Miyashita, 1976, Kumazawa & Fujii, 1984, Kasukawa & Fujii, 1985] This nervous control is responsible for the fast colour change, and permits fish to use changes of skin colour as a means of social signalling in either territorial, sexual, or aggressive encounters [Baker, 1993] The intensity of innervation, and accordingly the relative importance of nervous control, varies between different teleostean species Eel (*Anguilla anguilla*) melanophores, for example, are weakly innervated, whereas melanophores of the molly (*Poecilia latipinna*) and the European minnow (*Phoxinus laevis*) are well innervated [Pickford & Atz, 1957, Baker & Ball, 1975] Hormonal control of pigment migration, which takes minutes to days [Waring, 1963, Rodrigues & Sumpter, 1984], is slow compared to nervous control and important in long-term adaptation In the hormonal control of colour change two peptide hormones released from the pituitary are involved, namely α MSH and MCH This dual control is different from amphibians and reptiles where the mechanism of colour change is relatively simple and only α-MSH is involved [Bagnara & Hadley, 1973, Sherbrooke *et al*, 1988] α-MSH induces skin darkening by melanin dispersion, whereas MCH as an antagonist to α MSH induces melanin concentration and thereby paling of the skin α-MSH is produced in the melanotropes of the pituitary, while MCH is a neurohypophysial hormone which is produced in neurons of the ventral hypothalamus Communication between these two peptidergic cell types and between other hypothalamic neurons and pituitary melanotropes can be excellently studied in teleosts adapting to changes in background colour

CONTROL OF STRESS RESPONSE AND ION BALANCE IN FISH

Other important physiological processes in teleosts, regulated by neuro-endocrine communication via the hypothalamus-pituitary axis, are the stress response, and the control of ion balance The stress response can be described as a general reaction of a biological system to a diversity of stimuli, called stressors In teleosts, the regulation of the stress response occurs primarily via the hypothalamus-pituitary-interrenal (HPI) axis, and the most important factors involved are CRH, ACTH and cortisol In teleosts the control of ion balance and the stress response are intimately interrelated Many factors interfering with the ion

balance also evoke activation of the HPI axis, while during stress changes in ion balance very frequently occur [Eddy, 1981]

In fish, ionoregulatory control is very important, because the problems faced by most fish to maintain hydromineral homeostasis are substantial Freshwater teleosts are hyperregulators, thereby facing hyperhydration and diffusive ion losses, while seawater teleosts are hyporegulators maintaining their plasma osmotic value at a level of one third that of seawater, and face osmotic water losses and inflow of ions [Evans, 1979] Another environmental factor challenging the ion balance is acidification of the water, which may cause lowering of plasma ion levels The main sites of regulation of ion balance are the gills, intestine and kidney These organs regulate uptake and extrusion of mono- and divalent ions such as sodium, chloride, potassium and calcium These processes are under control of hormones from several glands Pituitary hormones directly involved in the regulation of ionic and osmotic homeostasis are PRL, GH, and possibly SL Also involved are cortisol from the interrenal cells of the head kidney, urotensin from the urophysis, and stanniocalcin, which is produced in the corpuscles of Stannius [Wendelaar Bonga, 1993]

The function of cortisol in the stress response as well as in the control of ion balance can be related to multiple actions of this corticoid in fish With respect to ion balance the mineralocorticoid action of cortisol dominates, while in response to stressful stimuli also its glucocorticoid actions and effects on immune competence [Maule *et al*, 1987] become noticeable The mineralocorticoid function of cortisol involves stimulation of proliferation of the ion-transporting cells of the gills and promotion of Na^+/K^+ ATPase activity of these cells, which is the driving force for monovalent ion transport [Dharmamba, 1979, Wendelaar Bonga, 1993] The glucocorticoid function of cortisol includes gluconeogenesis and protein catabolism

As mentioned above, cortisol functions in response to stressful stimuli as the end product of the HPI axis However, the described system is a simplified presentation of the actual control mechanism Other factors than ACTH (e g α-MSH [Lamers *et al*, 1992], GH, arginine vasotocin and catecholamines [Schreck *et al*, 1989]) can also directly or indirectly stimulate cortisol release, while most probably hypothalamic factors other than CRH, such as urotensin [Fryer *et al*, 1984] and MCH [Baker *et al*, 1985] also influence ACTH release

MELANIN-CONCENTRATING HORMONE

In the few teleost species investigated, MCH neurons are located in two hypothalamic regions A large number of magnocellular neurons is present in the nucleus lateralis tuberis (NLT), and

scattered neurons are found in the nucleus recessus lateralis (NRL) near the ventricular recess (Fig 1 1) Most hypothalamic axons project to the pituitary gland, where the peptide is stored and released into the circulation Other projections lead mainly to the ventral telencephalon and the optic tectum, but some axons project also to other regions of the brain [Baker, 1991] As mentioned above, the best known function of MCH is its melanin-concentrating effect on skin melanophores of teleosts However, from recent immunocytochemical and molecular cloning studies it became clear that MCH is not only synthesized in the hypothalamus of teleosts but also of higher vertebrates, including mammals In these vertebrates pituitary projections of MCH neurons are usually limited, while extensive projections throughout the central nervous system have been reported [Bittencourt, 1992] The function of mammalian MCH is less clear On basis of the axonal projections neuromodulatory functions in generalized arousal and sensorimotor integration have been suggested Moreover, MCH mRNA expression studies revealed that mammalian MCH neurons are responsive to stress, lactation, and osmotic stimuli such as salt loading and water deprivation [Baker, 1994] Very recently also for amphibians a role of MCH in osmo- and ionoregulation has been described [Smriga *et al*, 1994, Francis & Baker, 1995]

Sequence analysis of salmon [Ono *et al*, 1988, Minth *et al*, 1989] rat [Nahon *et al*, 1989], human [Presse *et al*, 1990] and mouse [Breton *et al*, 1993a] MCH cDNA revealed that MCH is synthesized from a preprohormone The MCH sequence appeared to be located at the carboxy terminus of the preprohormone Further analysis of the prohormone revealed the presence of other putative peptides that may be processed from the prohormone In salmon one putative peptide directly preceding MCH in the prohormone is called MCH gene related peptide (Mgrp) [Bird *et al*, 1990] In mammals two putative peptides are found The mammalian Mgrp analog is named neuropeptide glutamic acid-isoleucine (NEI) Preceding NEI another putative peptide is found, which dependent on its structure either is called neuropeptide glycine-glutamic acid (NGE) or neuropeptide proline-glutamic acid (NPE) Very recently it has been demonstrated that rat NEI is actually processed from the MCH preprohormone, and that it is released from cultured rat hypothalamic cells [Parkes & Vale, 1992] Functions of mammalian MCH and NEI appeared to be partly similar and partly different Both peptides inhibit oxytocin release *in vitro*, whereas only NEI inhibits vasopressin release from the rat pituitary [Parkes & Vale, 1993] The sites of action of MCH and NEI in the mammalian stress response have not been established yet The actual processing of teleost Mgrp is not yet demonstrated and also no functional studies with this putative peptide have been performed

Like in mammals, in teleosts a role for MCH in the control of the hypothalamus-pituitary interrenal axis involved in stress response has been suggested, since the CRH induced ACTH release from trout pituitaries is inhibited by MCH *in vitro* [Baker *et al*, 1985], and

trout adapted to white tanks appeared to have lower plasma cortisol levels [Baker & Rance, 1981, Green & Baker, 1991] and release less ACTH than black adapted fish [Gilham & Baker, 1985, Baker *et al*, 1986], differences which appear to be more pronounced under conditions of stress

AIM AND OUTLINE OF THE THESIS

The aim of this thesis was to study the structure and distribution of MCH precursor mRNA, and to investigate the role of MCH precursor-derived peptides in the control of background adaptation, the stress response and the regulation of ion balance in teleost fish Tilapia (*Oreochromis mossambicus*), a cichlid teleost, was chosen as experimental animal because of its impressive capacities to handle environmental challenges such as background colour [Van Eys, 1981], and osmotic challenges such as exposure to acidified water [Wendelaar Bonga & Balm, 1989, Lamers, 1994] and seawater [Balm, 1986, Verbost *et al*, 1994]

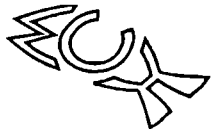
In *Chapter 2* the cloning and characterization of a cDNA encoding the tilapia MCH preprohormone is described It was shown that the putative tilapia Mgrp (tMgrp) is very different in length and amino acid sequence from other known Mgrp's and NEIs To study tissue distribution of tilapia preproMCH (ppMCH) mRNA in brain and peripheral tissues Northern blot analysis was performed

In *Chapter 3* the distribution of ppMCH mRNA and MCH immunoreactivity in brain and pituitary was studied in detail by Northern blot analysis and *in situ* hybridization MCH and its mRNA were localized in the NLT and NRL of the hypothalamus For the first time, the presence of ppMCH mRNA and MCH containing cells in cell bodies of the neurohypophysis could be demonstrated To study the ppMCH mRNA tissue content a quantitative dot blot analysis method was validated

In *Chapter 4* this quantitative ppMCH mRNA analysis method was used to study the possible differential response of MCH neurons of the NRL and NLT to environmental challenges that modulate the skin colour, the ion-balance and/or evoke a stress response To study the possible link of ppMCH mRNA expression with the stress response and ion balance, plasma cortisol, ACTH and chloride levels were determined as well

In *Chapter 5* the communication at the pituitary level between MCH neuronal fibers and melanotropes was investigated to gain better insight in the mechanism of action of MCH in background adaptation Therefore, the effect of MCH on α MSH release was studied quantitatively by *in vitro* superfusion experiments, and qualitatively by HPLC analysis of different α-MSH isoforms

In *Chapter 6* the biosynthesis and release of tMgrp was studied. An antiserum was raised against synthetic tMgrp and used for immunocytochemical analysis to establish tMgrp tissue distribution in the hypothalamus-hypophysial system. The biosynthesis and release of tMgrp was demonstrated by HPLC elution and a newly developed tMgrp ELISA using the tMgrp antiserum. Moreover, it was investigated whether tMgrp, like MCH, has a role in background adaptation by modulating pigment dispersion of the skin. Tilapia Mgrp appeared not to have an effect on pigment dispersion, and therefore in *Chapter 7* it was investigated whether this peptide could have a function at the pituitary level by examining its effects on *in vitro* α-MSH and ACTH release.



Chapter 2

Cloning and sequence analysis of hypothalamus cDNA encoding tilapia melanin-concentrating hormone

ABSTRACT

Melanin-concentrating hormone (MCH) is a neuroendocrine peptide involved in the regulation of skin pigmentation in teleosts We isolated and sequenced a 543 bp hypothalamic cDNA encoding the MCH preprohormone of tilapia (*Oreochromis mossambicus*) Initially, polymerase chain reaction (PCR) experiments were performed on hypothalamic RNA with a synthetic oligonucleotide primer corresponding to a conserved region of salmon and mammalian MCH peptide and an oligo (dT) primer A 0 2 kb PCR fragment was obtained and found to have low but significant nucleotide sequence similarity with the 3' ends of known MCH mRNAs Subsequently, the PCR fragment was used to screen λZAP cDNA libraries constructed from tilapia hypothalamic poly(A$^+$) RNA The cloned tilapia MCH preprohormone cDNA encodes a 133 amino acid protein of which 17 amino acids belong to the signal peptide The MCH peptide sequence is located at the carboxy terminus of the preprohormone structure and is preceded by a pair of arginine residues which can serve as a proteolytic cleavage site 23 to 25 amino acids further upstream in the prohormone structure three consecutive basic residues are present Cleavage at this site would yield a 22 amino acid MCH gene-related peptide (Mgrp), which is much larger than (13 amino acid) salmon and mammalian Mgrp A comparative structural analysis between tilapia preproMCH and its salmon and mammalian counterparts revealed that the MCH peptide sequence is very well conserved (100 % identity with salmon and 75 % identity with both rat and human MCH) In contrast, the remaining parts of the preproMCH structures have diverged considerably Northern blot analysis revealed the presence of tilapia preproMCH mRNA in the hypothalamus and not in other brain regions nor in several peripheral tissues

---

With M J Hut P H M Balm G J M Martens & S E Wendelaar Bonga
Fish Physiology and Biochemistry 11 117 124 (1993)

INTRODUCTION

Many lower vertebrates are capable of altering the pigmentation of their skin in response to variations in background coloration The physiological mechanism of background adaptation is of paramount importance for these animals and regulation of this process can occur by both neuronal and endocrine mechanisms In amphibians and reptiles one pituitary hormone, α-melanocyte-stimulating hormone (α-MSH), is involved in the control of skin pigmentation In teleost fish, however, a second peptide hormone, melanin-concentrating hormone (MCH), functions in this mechanism MCH is produced in the hypothalamus and most of the peptide is axonally transported to the pituitary, where it is stored in the neural lobe [Baker, 1991, Eberle, 1988] In addition to the pituitary projections, some MCH-containing axons project into the brain, where the role of MCH is still unknown Naito *et al*, 1985]

The primary structure of MCH was first determined following isolation from chum salmon (*Oncorhynchus keta*) pituitaries [Kawauchi *et al*, 1983] It appeared to be a cyclic heptadecapeptide Subsequently, the MCH peptide structure of other teleosts has been described Bonito (*Katsuwonas pelamis*) MCH appeared to be identical to salmon MCH, whereas only one amino acid substitution at the amino terminus was observed in case of the eel *Anguilla japonica* [Kawauchi, 1989] Recently, the structures of cDNAs encoding the MCH preprohormone in salmon, rat and man have been elucidated [Ono *et al* 1988, Minth *et al* 1989, Nahon *et al* 1989, Presse *et al*, 1990] and the MCH peptide has been purified from rat hypothalamic tissue [Vaughan *et al*, 1989] Hence, MCH is not only present in teleosts but also in other species where its function is yet unclear Regulatory roles in water balance and control of homeostatic functions [Presse *et al*, 1990, Zamir *et al*, 1986] or antagonistic effects on α-MSH-induced behaviour in rats [Eberle, 1988] have been proposed

At present little is known about the evolutionary conservation of the MCH preprohormone (ppMCH) in fish We therefore elucidated the structure of a tilapia hypothalamic mRNA that encodes the MCH precursor protein A comparative structural analysis between tilapia and other known MCH preprohormones is presented Furthermore, tissue specificity of tilapia ppMCH mRNA expression has been investigated by Northern blot analysis

MATERIALS & METHODS

Polymerase Chain Reactions

RNA of tilapia (*Oreochromis mossambicus*) (obtained from our laboratory stock) was prepared by the Nonidet P40 method [Sambrook *et al*, 1989], followed by purification of mRNA with oligo (dT) cellulose For polymerase chain reaction (PCR) analysis single stranded cDNA was synthesized from poly(A^+) RNA by using MLV reverse transcriptase (BRL) and 50 ng (dT) primer [CCTGCAGCGGCCGCATGCATTTTTTTTTTTTTTTT] in 1 x PCR buffer (Perkin Elmer Cetus), 1 mM dNTP, RNase inhibitor (19 U, Promega), 8 75 mM $MgCl_2$ in a final volume of 20 µl The template was amplified using 50 pmol each of a degenerate oligonucleotide primer corresponding to a conserved region of the MCH peptide [T(C/G)GGATCCGT(C/G)TA(C/T)(A/C)G(A/G)CC(A/C/G/T)TG(C/T)TGG] and the primer CCTGCAGCGCCGCATGCA for 30 cycles of denaturation (93 °C, 1 min), annealing (50 °C, 1 30 min) and extension (70 °C, 1 min) in a Perkin Elmer-Cetus Thermal Cycler with Ampli-Taq DNA polymerase (1 U, Perkin Elmer-Cetus) After agarose gel electrophoresis a 0 2 kb PCR fragment was extracted from the gel, digested with BamHI and NotI and ligated into a pBleuscript SK vector DNA sequencing was performed with T7 DNA polymerase by the dideoxy chain termination method [Sanger *et al*, 1977]

Construction of tilapia hypothalamus cDNA libraries

Two tilapia hypothalamus cDNA libraries were made The first one was constructed in the vector λZAPII according to the manufacturers instructions (Stratagene, la Jolla, CA) using 3 µg poly(A^+) RNA isolated with the Nonidet P40 method cDNA was synthesized and inserted into the EcoRI site of the λZAPII vector The cDNA library contained 2 x 10^5 independent clones

A second tilapia hypothalamus cDNA library was constructed with a ZAP-cDNA synthesis kit (Stratagene) using about 4 µg poly(A^+) RNA, isolated by the acid guanidinium thiocyanate-phenol-chloroform procedure [Chomczynski & Sacchi, 1987] cDNA was synthesized using an oligonucleotide that contained a poly (dT) sequence and a XhoI restriction site EcoRI adaptors were ligated and the cDNA was directionally cloned into the EcoRI XhoI sites of the Uni-ZAP XR vector This cDNA library contained 4 x 10^5 independent clones Both libraries were amplified

Screening of hypothalamus cDNA libraries

Replica nitrocellulose filters of the hypothalamus cDNA libraries were made The λZAPII library was screened at 42 °C in 6 x SSC (1 x SSC is 0 15 M NaCl, 15 mM sodium

citrate, pH 7 0), 1 % SDS, 40 mM sodium phosphate buffer, pH 7 0, 2 x Denhardt's solution, 0 1 % sodium pyrophosphate, 1 mM EDTA, 50 % formamide and 100 µg/ml herring sperm DNA Washing of filters was performed at room temperature (RT) and 60 °C in 2 x SSC, 0 1 % SDS, 0 1 % sodium pyrophosphate and 1 mM EDTA As a hybridization probe we used the 0 2 kb PCR fragment, labelled by nick translation according to standard procedures [Sambrook *et al*, 1989]

The Uni-ZAP XR library was screened at 45 °C in 5 x SSPE hybridization solution (5 x SSPE [1 x SSPE is 0 18 M NaCl, 10 mM sodium phosphate, pH 7 4, 1 mM EDTA], 5 x Denhardt's solution, 0 5 % SDS, 50 % formamide and 100 µg/ml herring sperm DNA) Washing was performed at RT and 60 °C until 0 1 x SSPE, 0 1 % SDS TMe58, a putative partial tilapia MCH cDNA clone isolated from the first library, was labelled by *in vitro* cRNA synthesis according to standard procedures [Sambrook *et al*, 1989] and used as a hybridization probe Hybridization positive phage plaques were purified and pBluescript DNA was prepared by *in vivo* excision according to the manufacturers protocol (Stratagene) DNA of both strands was sequenced

Northern blot analysis

Tilapia tissues were collected and immediately frozen on dry ice Total RNA from tilapia hypothalamus, brain without hypothalamus, ovary, intestine, liver, heart, skin, headkidney, was prepared with the acid guanidinium thiocyanate phenol-chloroform method RNA was run on a horizontal 1 % agarose gel in 2 2 M formaldehyde and MOPS buffer (0 02 M MOPS, 8 mM sodium acetate, pH 7 0, 1 mM EDTA) RNA was transferred to nitrocellulose filter and the filter was hybridized with a cRNA probe of TMe58 cDNA in 5 x SSPE hybridization solution with 50 % formamide at 45 °C for 16 h Washing was performed at RT and 60 °C until 0 1 x SSPE, 0 1 % SDS

RESULTS

PCR analysis

To obtain a tilapia MCH probe, PCR experiments were conducted on tilapia hypothalamus mRNA with a degenerate primer corresponding to ppMCH mRNA and an oligo (dT) primer The resulting 0 2 kb PCR fragment was cloned into pBluescript and sequenced The PCR fragment (not shown) had low but significant similarity with the 3' end of salmon ppMCH mRNA [Ono *et al*, 1988, Minth *et al*, 1989]

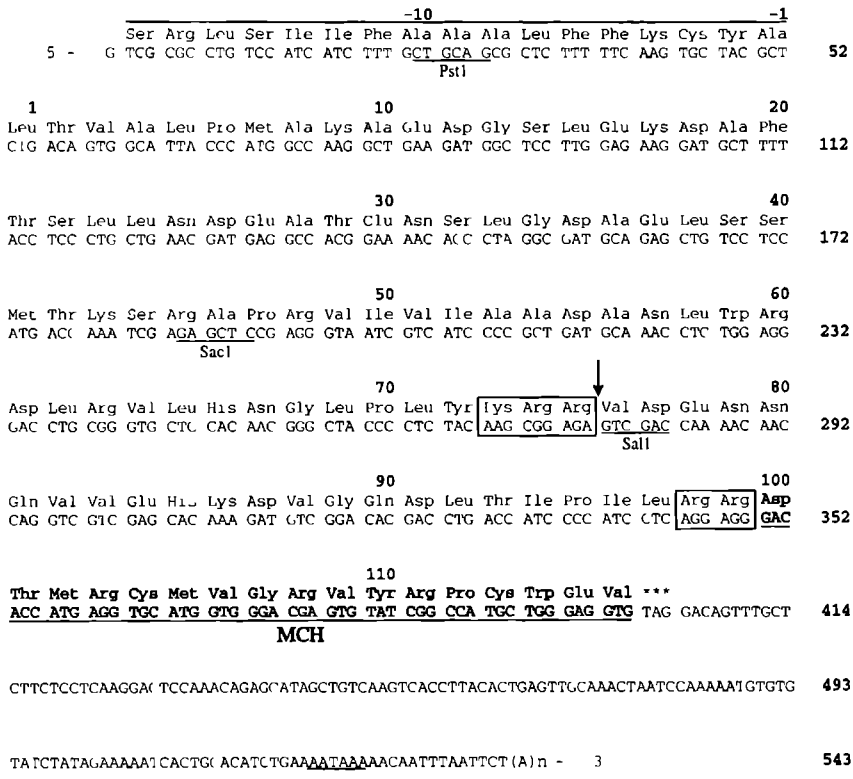


Figure 2.1 Nucleotide sequence and deduced amino acid sequence of a hypothalamic cDNA clone, TM16f encoding tilapia preproMCH The mature hormone MCH is underlined, the putative signal peptide sequence is overlined and the polyadenylation signal is underlined Putative proteolytic cleavage sites are boxed and restriction sites are underlined Numbering starts at the first amino acid of the prohormone The start of the partial cDNA clone TMe58 is indicated with an arrow

Tilapia ppMCH cDNA

Two tilapia hypothalamus cDNA libraries (2 x 10^5 and 4 x 10^5 clones respectively) were constructed and screened under high stringency conditions For screening of the first library the 0 2 kb MCH PCR fragment was used, which resulted in 12 hybridization-positive clones with the same 0 6 kb cDNA insert The sequence of this cDNA showed similarity in the 0 35 kb 3' part with salmon ppMCH mRNA [Ono *et al*, 1988, Minth *et*

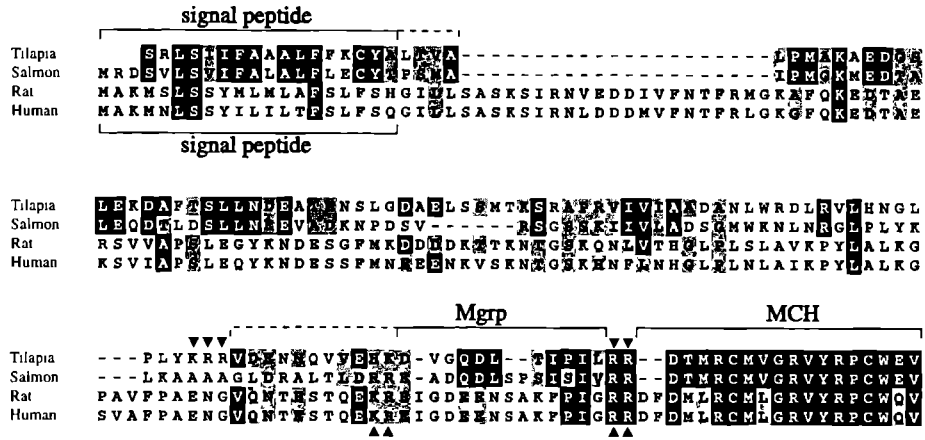


Figure 2.2 Alignment of the amino acid sequences of tilapia, salmon (sMCH1) [Minth et al., 1989], rat [Nahon *et al*, 1989] and human [Presse *et al*, 1990] MCH-preprohormones. The one-letter amino acid notation is used. Gaps (-) have been introduced to achieve maximum similarity. Residues identical with the tilapia preprohormone are indicated by black boxes; conserved amino acid substitutions are indicated by hatched boxes. The MCH peptide sequence, the putative MCH gene-related peptide (Mgrp) sequence (elongated by an interrupted line towards the putative tilapia cleavage site) are indicated. The putative signal peptide sequence of tilapia is overlined, the interrupted extension of the line indicates the salmon signal peptide cleavage site. The mammalian signal peptide sequence is underlined. Arrowheads indicate potential recognition sites for proteolytic cleavage enzymes.

al., 1989]. The 5' end was very AT rich and contained several stop codons (not shown). A SalI fragment of the putative MCH prohormone coding part was subcloned after removing the poly(A) tail (TMe58). This clone was used to screen a second hypothalamus Uni-ZAP XR cDNA library. Two hybridization positives were purified. The longest cDNA clone (clone TM16f, size of 0.58 kb) was selected for further analysis. Figure 2.1 shows the nucleotide sequence and deduced amino acid sequence of TM16f cDNA. The longest open reading frame codes for 133 amino acids. The MCH peptide is located at the carboxy-terminus of the preprohormone, preceded by two arginine residues, which are proposed to function as cleavage site for proteolytic processing to mature hormones [Harris, 1989]. Three consecutive basic amino acid residues are found at amino acids 72-74 and cleavage at this site could produce a 42 amino acid peptide or a 22 amino acid peptide and mature MCH. At the amino-terminus a sequence with characteristics of a signal peptide is present. The most probable site of signal peptide cleavage is at alanine -1 [Heijne, 1986; Perlman & Halvorson, 1983], yielding a 116 amino acid prohormone with a calculated molecular

mass of 13,091 D The 3' non-coding region of the cDNA contains 20 to 15 bp upstream of the poly(A) tail a polyadenylation consensus sequence [Proudfoot & Brownlee, 1976]

TMe58 cDNA was identical to the corresponding part of TM16f Comparison of the amino acid sequence deduced from TM16f with salmon and mammalian ppMCH structures (Fig 2 2) showed a high degree of identity at the carboxy terminus where the MCH peptide is located (100 % and 75 % identity, respectively) The amino acid sequence identity between TM16f and the salmon MCH preprohormone at the amino terminus in the putative signal sequence was also high (65 % identity, Fig 2 2) We therefore conclude that TM16f cDNA encodes a tilapia MCH preprohormone

Northern blot analysis

Northern blot analysis of total RNA isolated from a variety of tilapia tissues with tilapia proMCH cDNA clone TMe58 as a probe revealed a single band at about 850 bases for hypothalamus RNA, whereas for brain (minus hypothalamus), ovaria, intestine, liver, heart, skin, headkidney (Fig 2 3A), fin and muscle RNA (data not shown) no signal could be detected

DISCUSSION

Comparison of tilapia, salmon and mammalian MCH preprohormones

In this report we describe the cloning and expression of tilapia ppMCH mRNA A structural comparison of the tilapia MCH preprohormone with its salmon counterpart [Ono *et al*, 1988, Minth *et al*, 1989] shows a high identity in the MCH peptide and the signal peptide sequence (100 % and 65 % amino acid sequence identity respectively) In the remaining part the similarity between the proMCH structures of the two teleost fishes is remarkably low, with just 26 % amino acid sequence identity and 53 % similarity Thus, except for the MCH and signal peptide regions the MCH preprohormones of cichlids (tilapia) and the more primitive salmonids have diverged considerably during evolution

The similarity between tilapia and mammalian MCH preprohormones [Nahon *et al*, 1989, Presse *et al*, 1990] is very low, except in the MCH peptide part, where 75 % amino acid sequence identity occurs It can therefore be concluded that in general the MCH preprohormone is a very poorly conserved peptide precursor, with only high identity in the MCH peptide coding part Since functionally significant protein regions as a rule are strongly conserved during evolution, it is obvious that the MCH part of the MCH preprohormone has substantial physiological importance, although species specific

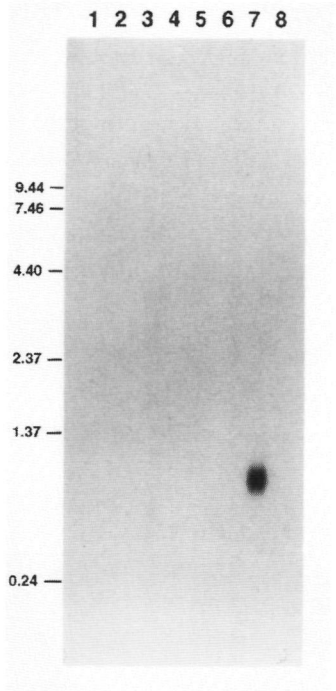


Figure 2.3 Northern blot analysis of tilapia preproMCH mRNA. Thirty microgram (unless otherwise mentioned) per sample of total RNA was subjected to electrophoresis on an 1 % agarose gel (20 mM MOPS buffer, 2.2 M formaldehyde), transferred to a nitrocellulose filter and hybridized with a tilapia proMCH (TMe58) cRNA probe. Lanes 1, ovaria; 2, intestine; 3, liver; 4, heart; 5, skin; 6, headkidney; 7, hypothalamus (20 µg of RNA); 8, brain without hypothalamus. Positions of RNA size-markers are indicated.

functions of the less well conserved regions cannot be ruled out. Poor conservation of non-biologically active peptide regions of prohormones has been reported for other hypothalamic neuropeptide preprohormones like the CRH precursor [Okawara *et al.*, 1988].

When the sequence comparison of the tilapia and the salmon MCH preprohormones is studied in more detail, some marked differences are observed (Fig. 2.2). First, based on accepted criteria [Heijne, 1986; Perlman & Halvorson, 1983], the putative cleavage site of the signal peptide of the tilapia MCH preprohormone is located four amino acids more amino-terminal than the putative salmon cleavage site, although in tilapia an alanine residue corresponding to the salmon cleavage site also lies in a favorable position for cleavage. It has to be investigated which of the potential sites in tilapia is actually used. Second, from the carboxy-terminal part just preceding the MCH peptide in the tilapia prohormone structure a 22 residue peptide could be cleaved off by using a potential proteolytic cleavage site consisting of three consecutive basic amino acid residues. In salmon, trout [Bird *et al.*, 1990] and mammals [Nahon *et al.*, 1989; Presse *et al.*, 1990], however, a smaller 13 residue or 1.7 kD peptide, called MCH gene-related peptide (Mgrp; Bird *et al.*, 1990] can be formed. At the cleavage site of this smaller peptide a single

lysine residue is present in the tilapia precursor Since monobasic residues can also serve as cleavage sites [Schwartz, 1986] it cannot be excluded that in tilapia a small 11 amino acid peptide is cleaved off The functional relevance of the putative 11 or 22 amino acid peptides is still unclear

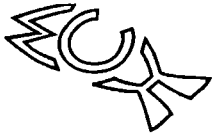
Tilapia most probably possesses only one MCH precursor gene, since Southern blot analysis of genomic DNA digested with several restriction enzymes that do not cut in the cDNA corresponding to the proMCH cRNA probe, yields only one band per lane (data not shown) The presence of one gene in tilapia is in contrast with the existence of two genes in salmonids, but this difference can be explained by the fact that salmonids are tetraploid [Ohno *et al*, 1968] and cichlids diploid

Comparison of the tilapia MCH preprohormone with the ANP and the *Aplysia* peptide A preprohormone

It has been reported that the mammalian MCH preprohormone shows significant amino acid sequence identity with the precursor for *Aplysia* peptide A (ppPep A) [Nahon *et al* 1989, Presse *et al*, 1990] and human preproatrial natriuretic peptide (ppANP) [Presse *et al*, 1990] These observations led to the suggestion that ppMCH, ppPep A and ppANP are evolutionarily related, which also might imply some functional relationship Since both ANP and *Aplysia* Pep A are involved in regulation of water balance and vascular functions [Scheller *et al*, 1984, De Bold, 1985] a similar role has been proposed for mammalian MCH [Presse *et al*, 1990, Baker, 1991] Computer searches (using the Pearson and Lipman program) of a NBRF protein data base for homologies of tilapia ppMCH with other sequences did not reveal similarities with either ppANP or ppPep A When we subsequently compared tilapia ppMCH with human ppANP, eel ANP [Takei *et al*, 1989] and *Aplysia* ppPep A, the low degree of resemblance was confirmed The identity between the last 13 amino acids of tilapia MCH and human ANP was 31 % (versus 38 % if the human peptides are compared) [Presse *et al* 1990], whereas identity between tilapia MCH and the corresponding part of eel ANP was only 23 % In the signal peptides the identity between tilapia ppMCH and human ppANP is 24 % (versus 37 % in the human signal peptides) [Baker, 1991] The percentages of identity are even lower if tilapia ppMCH and *Aplysia* ppPep A are compared (18 % identity in the MCH coding region, 21 % in 19 amino acids of the *Aplysia* Pep A coding part and 6 % in 17 amino acids of the signal peptides) In addition, it has been observed before that the similarity between salmon ppMCH and *Aplysia* ppPep A is also very low [Baker, 1991] Taken together, these findings suggest that there has been no extensive selective pressure in teleosts to maintain structural conservation of these three peptide precursors

Tissue distribution of tilapia ppMCH mRNA

The preliminary findings of a strong ppMCH mRNA signal in hypothalamic tissue while not in other brain areas or peripheral tissues are in line with the tissue distribution reported for salmon ppMCH mRNA [Ono *et al*, 1988, Minth *et al*, 1989] *In situ* hybridization and dot blot studies are in progress to determine the exact location of tilapia ppMCH mRNA and to investigate ppMCH mRNA expression in animals adapted to a number of environmental challenges such as changes in background colouration



Chapter 3

Expression of tilapia prepro-melanin-concentrating hormone mRNA in hypothalamic and neurohypophysial cells

ABSTRACT

Melanin-concentrating hormone (MCH) is a neuropeptide involved in background adaptation in teleost fish, and in multiple regulatory functions in mammals and fish To study the expression of the MCH preprohormone (ppMCH) in teleosts, we first cloned a hypothalamic cDNA encoding the complete ppMCH of tilapia (*Oreochromis mossambicus*), and a cRNA probe derived from a 270 bp ppMCH cDNA fragment was used for the expression studies The level of ppMCH mRNA expression in tilapia hypothalamus, measured by dot blot analysis, was significantly higher in fish adapted to a white background than in black-adapted animals, which is in accordance with the reported MCH plasma and tissue concentrations in fish Northern blot analysis not only revealed a strong ppMCH mRNA signal in the hypothalamus, but also the presence of ppMCH mRNA in the neurointermediate lobe (NIL) of the pituitary *In situ* hybridization and immunocytochemistry showed that ppMCH mRNA as well as MCH immunoreactivity are located in perikarya of two hypothalamic regions, namely in the nucleus lateralis tuberis (NLT) and the nucleus recessus lateralis (NRL) Quantitative analysis by dot blot hybridization revealed about eight times more ppMCH mRNA in the NLT than in the NRL and NIL of mature tilapias ppMCH mRNA in the NIL could be localized to cell bodies of the neurohypophysis, which were also MCH immunoreactive

With E R M Eckhardt A J M Coenen G J M Martens P H M Balm & S E Wendelaar Bonga
Journal of Molecular Endocrinology 14 199 207 (1995)

INTRODUCTION

Melanin-concentrating hormone (MCH) is a cyclic neuropeptide first isolated from chum salmon pituitary extracts [Kawauchi *et al*, 1983] More recently the peptide has been identified in rat hypothalamus [Vaughan *et al*, 1989] Concerning MCH function and distribution, several differences exist between teleosts and mammals Firstly, some of the biological functions of MCH appear to be different in fish and mammals In teleosts, but not in mammals, MCH is a hormone involved in background adaptation by acting at the skin and pituitary [Eberle, 1985, Baker, 1991] Another physiological function attributed to MCH is a role in the control of lactation and fluid homeostasis in mammals, an observation not yet documented in fish [Zamir *et al*, 1986, Knollema *et al*, 1993, Parkes & Vale, 1993] In both teleosts and mammals MCH could play a role in the stress response by modulating the activity of the hypothalamic-pituitary-adrenal (HPA) axis The second difference between these vertebrate classes concerns the control mechanism and site of action in the stress response It has been reported that in teleosts MCH acts at the pituitary level by inhibiting the release of ACTH [Baker *et al*, 1985, 1986] In mammals it has been considered unlikely that MCH acts on the ACTH cells, since *in vitro* no effect of rat MCH was found on ACTH secretion from rat corticotropes [Navarra *et al*, 1990] It has further been demonstrated *in vivo* that MCH indirectly can stimulate ACTH secretion from rat pituitaries by acting at the level of the central nervous system, primarily via a CRH-dependent pathway [Jezova *et al*, 1992] In contrast, in teleost fish MCH inhibits CRH release from the hypothalamus *in vitro* [Baker, 1991] The level of synthesis and secretion of MCH in response to stress also seem to be different in teleosts and mammals The secretion of MCH can be enhanced by repeated exposure to stressors in rainbow trout [Green & Baker, 1991], whereas MCH gene expression in the rat appears to be decreased by chronic stress [Presse *et al*, 1992] Finally, the tissue distribution of MCH is different in teleosts and mammals In teleost fishes, MCH is synthesized in perikarya of the ventral hypothalamus and most axons release the peptide in the neurohypophysis Other fibers project into several regions of the brain In mammals and other non teleostean vertebrates MCH-producing perikarya are located more centrally in the hypothalamus and the majority of axons project to various brain regions, whereas in general only few projections lead into the pituitary [Eberle, 1985, Baker, 1991]

Knowledge of the amino acid sequence of MCH allowed several research groups to elucidate the sequences of cDNAs encoding MCH preprohormones (ppMCH) from salmon [Ono *et al*, 1988, Minth *et al*, 1989 Nahon *et al*, 1991], rat [Nahon *et al*, 1989], man [Presse *et al*, 1990] and mouse [Breton *et al*, 1993a] In addition to the MCH peptide, a second potential cleavage product preceding MCH in the prohormone structure was

postulated This peptide was called MCH gene-related peptide (Mgrp) in fish [Bird *et al*, 1990, Baker, 1991], and neuropeptide E-I (NEI) in mammals [Nahon *et al*, 1989] Recently, evidence has been provided that this novel neuropeptide is actually processed from the MCH preprohormone in mammals [Parkes & Vale, 1992]

We recently reported the cloning of a partial hypothalamic ppMCH cDNA of the tilapia (*Oreochromis mossambicus*), an advanced teleost [Groneveld *et al*, 1993] In the present study we cloned a hypothalamic cDNA encoding the complete structure of the tilapia MCH preprohormone To determine the synthetic activity of MCH perikarya in fish in response to changes in their environment, we measured the level of ppMCH mRNA expression in tilapias adapted to different backgrounds Moreover, we examined the distribution of tilapia ppMCH mRNA Surprisingly, we localized ppMCH mRNA not only in the hypothalamus but also in the neurohypophysis

MATERIALS & METHODS

Animals

Freshwater tilapias, *Oreochromis mossambicus*, of both sexes were bred in our laboratory and fed on a commercial dried fish food (Tetramin) The fish were kept in aquaria with Nijmegen tap water at 28 °C, under a 12 h light, 12 h dark cycle Male tilapias weighing between 15 and 20 g were adapted to black and white backgrounds by transferring them from glass aquaria (neutral background') to plastic black and white tanks, respectively The tanks contained 80 l of tap water The animals were kept for two weeks in these tanks, and were fed daily The animals were sacrificed by spinal transection and the hypothalami were dissected from the brain In order to separate the nucleus lateralis tuberis (NLT) and nucleus recessus lateralis (NRL), a transverse incision was made from the ventral side of the hypothalamus just caudal of the pituitary to the dorsal side of the optic chiasm

Construction and screening of tilapia hypothalamus cDNA library

A tilapia hypothalamus cDNA library was constructed with a λZAP-cDNA synthesis kit (Stratagene) using about 4 µg poly(A^+) RNA RNA was isolated by the acid guanidinium-thiocyanate-phenol chloroform procedure [Chomczynski & Sacchi, 1987], followed by purification of poly (A^+) RNA with an oligo (dT) cellulose column (Stratagene), according to the manufacturers instructions cDNA was synthesized using an oligonucleotide that contained a poly dT sequence and a XhoI restriction site EcoRI adaptors were ligated and the cDNA was directionally cloned into the EcoRI-XhoI sites of the Uni ZAP XR vector

This library contained 2 x 10^5 clones Replica nitrocellulose filters (Schleicher & Schuell) of 60,000 clones of the primary hypothalamus cDNA library were made The filters were screened at 45 °C in 5 x SSPE hybridization solution (5 x SSPE [1 x SSPE is 0 18 M NaCl, 0 01 M NaH_2PO_4, pH 7 4, 1 mM EDTA], 5 x Denhardt's solution [0 1 % polyvinylpyrrolidine, 0 1 % bovine serum albumine, 0 1 % Ficoll 400], 0 5 % SDS, 50 % formamide and 100 µg/ml herring sperm DNA) Washing was performed at room temperature (RT) in 1 x SSPE, 0 1 % SDS, and subsequently for 20 min at 60 °C in 1 x SSPE, 0 1 % SDS, 15 min at 65 °C in 0 25 x SSPE, 0 1 % SDS and 15 min at 65 °C in 0 1 x SSPE, 0 1 % SDS The insert of the partial tilapia MCH-cDNA clone TM16f [Groneveld *et al*, 1993] was labeled with ^{32}P by random priming according to standard procedures [Sambrook *et al*, 1989] The labeled insert was used as a hybridization probe Hybridization-positive phage plaques were purified, and pBluescript DNA was prepared by *in vivo* excision according to the manufacturers protocol (Stratagene) DNA sequencing was performed with T7 DNA polymerase and the dideoxy chain termination method [Sanger *et al*, 1977]

ppMCH mRNA analysis

For dot blot analysis, total RNA samples, isolated by the acid guanidinium-thiocyanate phenol chloroform procedure, were resuspended in 300 to 1000 µl of a solution containing 5 x SSPE, 7 4 % formaldehyde After denaturing by heating for 10 min to 65 °C, and then cooling on ice, RNA was blotted on nitrocellulose filters using a dot blot apparatus (Bio Rad) Filters were baked at 80 °C for 2 h and hybridized in 5 x SSPE hybridization solution with a ^{32}P labeled cRNA probe of clone TMe58 This clone contains a 270-bp tilapia MCH cDNA encoding part of the prohormone and the 3' non translated region [Groneveld *et al*, 1993] Washing was performed at RT and 65 to 68 °C until 0 1 x SSPE, 0 1 % SDS Levels of ppMCH mRNA were quantified by densitometric scanning of the autoradiograms Prior to blotting, control samples were RNase treated by resuspending RNA pellets in 30 µl RNase digestion mixture (10 mM TrisHCl, pH 7 5, 5 mM EDTA, 300 mM NaCl, 0 8 µg RNase A (Pharmacia), 17 5 units RNase T1 (Boehringer)), followed by 1 5 h incubation at 37 °C

For Northern blot analysis, total RNA was run on a horizontal 1 % agarose gel in 2 2 M formaldehyde and morpholino-propanesulfonic (MOPS) buffer (0 02 M MOPS, 8 mM sodium acetate, pH 7 0, 1 mM EDTA) RNA was transferred to a nitrocellulose filter, and hybridized as described above To control integrity of the RNA, hypothalamus and liver RNA samples of the same isolation were stained with ethidium bromide after gel-electrophoresis

Immunocytochemistry and *in situ* hybridization

Brains or pituitary glands, obtained from tilapias (20-80 g in weight) kept at a neutral background, were fixed overnight in Bouin-fluid, dehydrated and embedded in paraffin Five µm sections were mounted on poly L-lysine coated microscope slides Alternating sections were used for either immunocytochemistry or *in situ* hybridization

For immunocytochemistry slides were incubated at RT (unless mentioned otherwise) with 0 05 M Tris buffered saline (TBS), containing 0 3 % Triton for 5 min (two times), and 20 % normal goatserum in TBS/Triton for 10 min This was followed by incubation with MCH antiserum (kindly provided by Dr B I Baker) [Barber *et al*, 1987] diluted 1 4000 in TBS/Triton, for 16 h at 4 °C The MCH antiserum also recognizes the MCH prohormone [Bird *et al*, 1990] Then the slides were subsequently incubated with goat anti rabbit IgG (Nordic Immunology, Tilburg), diluted 1 150 in TBS/Triton, for 60 min, and rabbit PAP (Nordic), diluted 1 1000 in TBS/Triton, for 60 min Finally, slides were treated with 0 025 % 3,3 diaminobenzidine, 0 25 % nickle ammonium sulphate, and 0 01 % H_2O_2 in 0 05 M TrisHCl, pH 7 6 For specificity control the MCH antiserum was preadsorbed with 1 µM synthetic MCH (Peninsula) prior to incubation on the slides

In situ hybridization was performed following a procedure slightly modified from the one described by Tensen *et al* [1991] Briefly, sections were digested with 0 1 % pepsine, post fixed with 2 % paraformaldehyde in PBS, and rinsed in 1 % hydroxylammonium and PBS Dehydrated slides were hybridized with 150 µl hybridization buffer containing the probe (see below), 4 x SSC, 5 x Denhardt's solution, 50 % formamide, 10 % dextran sulphate and 200 µg/ml yeast tRNA, at 50 °C for 16 hr After hybridization slides were washed in 2 x SSC (2 times 30 min), 1 x SSC (30 min) and 0 5 x SSC (2 times 30 min) at 37 °C Finally, sections were processed for detection of digoxigenin Digoxigenin labeled cRNA derived from the 270-bp tilapia ppMCH cDNA fragment of clone TMe58 was used as antisense probe To assess the specificity of the hybridization signal a 231 bases sense probe was used Probes were labeled with digoxigenin, using a labeling and detection kit (Boehringer) according to the manufacturer's instructions Briefly, probes were labeled by cRNA synthesis by incorporation of digoxigenin-labelled uridine triphosphate (5 ng labeled cRNA/ 150 µl hybridization buffer) After hybridization to the target mRNA hybrids were detected by enzyme linked immunoassay using anti digoxygenin/alkaline phosphate conjugate and subsequently enzyme catalyzed colour reaction with 5-bromo-4-chloro-3 indolyl phosphate (X-phosphate) and nitroblue tetrazolium salt (NBT) Sections were examined with bright field microscopy Nomenclature of brain areas was based on the work of Batten and coworkers [Batten *et al* 1990]

Data analysis

Data are presented as the mean ± SEM. For statistical analysis the Student's *t* test was used. Significance was accepted at $P < 0.05$.

RESULTS

Analysis of hypothalamic cDNA encoding tilapia ppMCH

A primary tilapia hypothalamus cDNA library (60,000 clones) was screened under high-stringency hybridization conditions with TM16f, a partial tilapia ppMCH cDNA encoding proMCH and a portion of the signal peptide [Gröneveld *et al.*, 1993]. Ten hybridization-positive clones were isolated. Three of them were selected for further analysis. Two clones appeared to contain a full-length tilapia ppMCH cDNA encoding a 136-amino acid precursor with a calculated M_r of 15,410. Both full-length tilapia ppMCH cDNA clones were identical to the corresponding part of TM16f. The complete tilapia ppMCH cDNA sequence has been assigned accession number X81144 O. mossambicus mRNA in the EMBL Data Library. The full amino acid sequence of the putative tilapia ppMCH signal peptide is MRQSRLSIIFAAALFFKCYALTVA. Note that Ala^{20} in this sequence also follows accepted criteria [Heijne, 1986] for signal peptide cleavage.

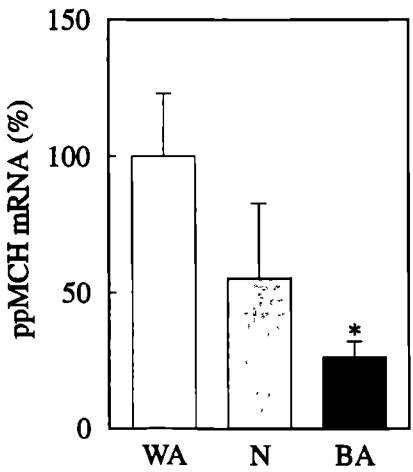


Figure 3.1 Levels of ppMCH mRNA in the hypothalamus of tilapia adapted to a white background (WA, open bar, n = 7), a neutral background (N, grey bar, n = 4), and a black background (BA, black bar, n = 8). ppMCH mRNA levels were determined by dot blot analysis. Signals were quantified by densitometric scanning of the autoradiogram. The relative ppMCH mRNA levels are expressed as percentage of the level in white-adapted tilapia. *, $P < 0.01$ if compared with white-adapted animals. The experiment was performed three times with comparable results.

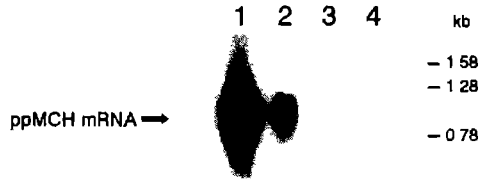


Figure 3.2 Northern blot analysis of tilapia RNA Total RNA was subjected to electrophoresis on an 1 % agarose gel, transferred to a nitrocellulose filter and hybridized with a tilapia pMCH (TMe58) cRNA probe The blot was exposed for 40 hrs with intensifying screens RNA was extracted from, lane 1, hypothalamus (~ 20 µg, corresponding to 2 hypothalami), 2, neurointermediate lobe of pituitary (~ 7 µg, 4 NILs), 3 rostral pars distalis of pituitary (~ 4µg 4 RPDs), 4, liver (~ 25 µg)

Quantification of hypothalamic ppMCH mRNA expression

In order to measure relative ppMCH mRNA levels in tilapia hypothalami in response to environmental changes a dot blot analysis assay was developed ppMCH mRNA hybridization signals of dot blotted hypothalamic RNA samples from individual fish were measurable after one to three days of autoradiography In RNase-treated hypothalamus RNA samples no ppMCH mRNA signal was detectable Specificity of the proMCH cRNA probe was tested by Northern blot analysis at the same hybridization conditions Only one band of 0 9 kb was obtained (not shown, see also Fig 3 2) Analysis of hypothalamic RNA of tilapias adapted for two weeks to black or white backgrounds revealed significantly more ppMCH mRNA in the hypothalamus of white-adapted fish than in the hypothalamus of black adapted animals Tilapias kept in glass aquaria ('N" in Fig 3 1) were found to contain intermediate ppMCH mRNA levels relative to black- and white-adapted fish

Tissue distribution

Northern blot analysis of total RNA isolated from a number of tissues revealed in the hypothalamus a band of 0 9 kb, whereas for liver and rostral pars distalis (RPD) of the pituitary no signal was obtained Surprisingly, the neurointermediate lobe (NIL) of the tilapia pituitary also contained ppMCH mRNA, although per animal the amount of MCH mRNA in this tissue was much less than in the hypothalamus (Fig 3 2) Note that different quantities of RNA derived from different numbers of animals were loaded in each lane

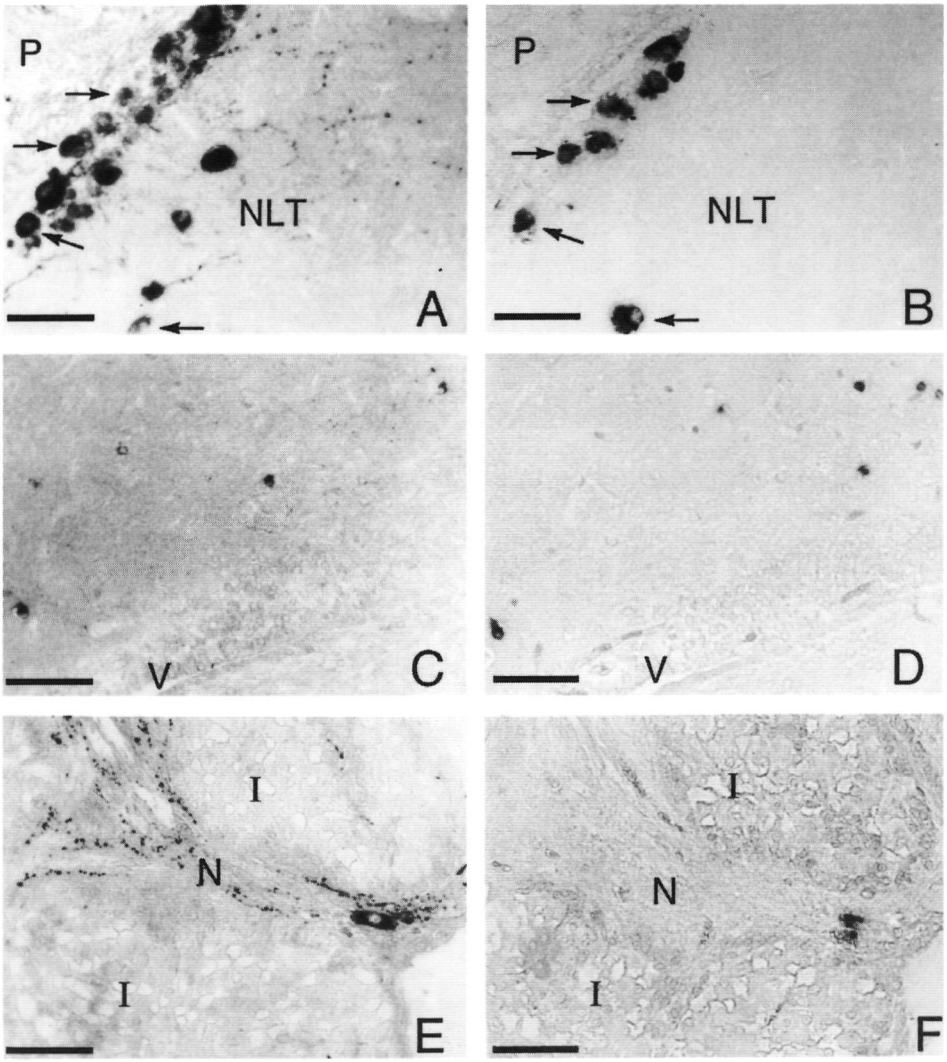


Figure 3.3 Immunocytochemical and *in situ* localization of tilapia MCH and ppMCH mRNA. x 300, scale bars are 50 μm. **A** and **B**, **C** and **D**, **E** and **F** are alternating sections. **A**, **C**, and **E** are stained with MCH-antiserum, in **B**, **D** and **F** ppMCH mRNA is shown by non-radioactive *in situ* hybridization. **A**, **B**, bottom of ventral hypothalamus; magnocellular neurons (arrows), containing MCH and ppMCH mRNA are found in the NLT. Note that cells immunostained with MCH-antiserum (**A**) are stained for ppMCH mRNA as well(**B**; indicated by arrows). **C**, **D**, smaller cells occur near the lateral ventricle. **E**, **F**, pituitary; MCH immunostaining is visible in the neurohypophysis, predominantly in axons and also in a cell body. Only the cell body is also labeled for ppMCH mRNA (**F**), whereas no ppMCH mRNA is detectable in axons. Abbreviations: NLT, nucleus lateralis tuberis; P, pituitary; V, lateral ventricle; N, neurohypophysis; I, intermediate lobe.

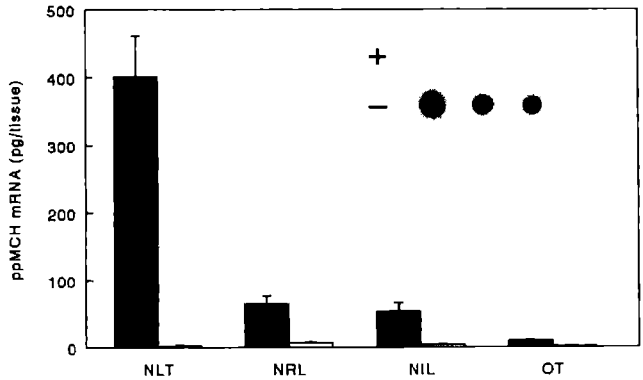


Figure 3.4 Dot blot quantification of ppMCH mRNA in different brain and pituitary regions. Total RNA samples of tissues of individual mature male tilapia's (108 ± 11 g in weight, n = 5) from neutral background were divided into two portions. One was blotted without further treatment (filled bars), the other was RNase treated before blotting (open bars). The amount of total RNA on the blot was 1 µg for NLT (derived from a quarter of the total tissue of one animal), 1 µg for NIL, and 5µg for NRL (both derived from half of the tissue of one fish) and 5 µg for optic tectum (OT) as a negative control. The blot, containing a sense pMCH cRNA standard dilution series, was hybridized with a pMCH cRNA probe Hybridization signals were quantified by densitometric scanning of the autoradiogram The values were converted by a sense pMCH cRNA standard curve to picograms of ppMCH mRNA per tissue of one animal. A representative autoradiograph showing ppMCH mRNA in respectively NLT, NRL, NIL and OT is displayed in the inset. The row of RNase treated samples is indicated with +, the untreated samples with -. Note that in case of NLT only half of the sample was loaded on blot.

Immunocytochemical analysis revealed MCH-containing perikarya in the ventral hypothalamus. Two groups of cells could be distinguished. The biggest group of large cell bodies, 12-20 µm in diameter, was located ventrally in the hypothalamus in the NLT (Fig. 3.3A). A second population of scattered small cell bodies (4-6 µm in diameter) was found near the lateral ventricle in the NRL (Fig. 3.3C). A few MCH-containing cell bodies (about 12 µm in diameter) were observed in the neurohypophysis (Fig. 3.3E). However, the majority of staining in the neurohypophysis was found in axonal nerves derived from MCH-containing hypothalamic neurons (Fig. 3.3E). MCH-containing fibers were also observed in several parts of the brain. Most were found in the hypothalamus and in the ventral telencephalon, whereas some others were detected in the optic tectum. When antiserum preadsorbed with synthetic MCH was used, no staining was found in brain and pituitary (not shown).

The anatomical distribution of ppMCH mRNA was investigated by *in situ* hybridization using an anti-sense pMCH cRNA probe The sections used for *in situ* hybridization alternated with sections used for immunocytochemistry ppMCH mRNA was detected in hypothalamic cell groups corresponding to areas stained by MCH-antiserum In neurons of the NLT both MCH immunostaining and ppMCH mRNA were present in the same cell bodies (Figs 3 3AB) Because of the small size of the cells in the NRL, it was not possible to stain the same cells of this region in alternating sections However, as shown in Figs 3 3CD, small MCH-producing cells were detected in the NRL with MCH immunostaining as well as with *in situ* hybridization In accordance with the Northern blot results, ppMCH mRNA was observed in some regions of the neurohypophysis Neurohypophysial ppMCH mRNA could be located in the same cell bodies that were immunostained for MCH (Figs 3 3EF) These cells often were located in the dorsal part of the neurohypophysis, as shown in Fig 3 3E and F, but occasionally they were also observed in the part of the neurohypophysis that penetrates deeply into the intermediate lobe No staining for ppMCH mRNA was observed in axonal fibers in the brain and pituitary gland, or when a sense control probe was used (not shown)

In order to study the relative amounts of ppMCH mRNA in the NLT and the NRL, tilapia hypothalami were divided into two parts by a transverse incision The actual separation of the cell groups was confirmed by MCH immunocytochemistry on sagittal brain sections of three animals (not shown) To be able to measure ppMCH mRNA levels in individual hypothalamic and hypophysial regions, the above mentioned dot blot analysis assay had to be refined By using a sense MCH cRNA standard curve the detection limit was found to be about 1 pg ppMCH mRNA/dot The signal in a negative control of optic tectum tissue of equal weight to NRL tissue, was significantly lower (5 times) than in NRL ($P < 0.01$) and NIL samples ($P < 0.025$) Furthermore, hybridization signals of RNase-treated samples were around the detection limit (Fig 3 4) Using this assay the majority of ppMCH mRNA was measured in the NLT region (400 pg/fish, Fig 3 4), about eight times more than in the NRL and NIL

DISCUSSION

In this study we cloned a hypothalamic cDNA encoding the complete structure of tilapia ppMCH Comparison of the tilapia MCH prohormone with its salmon and mammalian counterparts showed that only the MCH peptide is well conserved during evolution, while Mgrp and NEI are remarkably poorly conserved among the species examined [Groneveld

et al, 1993] Cloning of the complete tilapia MCH preprohormone mRNA allowed us to compare the signal peptide sequences The amino acid sequence identity between the tilapia and salmon (MCH-1) [Minth *et al*, 1989] signal peptides is considerable (58 %), whereas the identity between the tilapia and human [Presse *et al*, 1990] signal peptides is much lower (17 %)

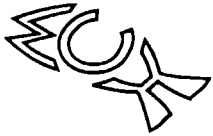
For quantification of ppMCH mRNA levels we developed a dot blot assay because of its sensitivity and simplicity [Van Tol & Burbach, 1989] With this method significantly more ppMCH mRNA was found in hypothalami of tilapia adapted to a white background than in black-background adapted fish The difference in ppMCH mRNA level supplements the reported differences in synthetic activity of MCH neurons of teleosts kept on black and white backgrounds Morphological studies in carp indicated that MCH producing cells are more active in white-adapted fish These cells have larger cytoplasmic and nuclear areas and more prominent nucleoli than in black-adapted carps [Bird & Baker, 1989] Recently, it was shown that hypothalamic *de novo* MCH synthesis of trout adapted to a white background is doubled if compared with black background adapted fish [Baker & Bird, 1992] The levels of hypothalamic ppMCH mRNA and MCH synthesis are in accordance with MCH secretion It is known that more MCH is present in the circulation of white-adapted trouts than in black-adapted animals, whereas the rate of MCH secretion in eel and carp also differs in response to changes of background colouration, as judged by the MCH content of the pituitary gland [Baker, 1991]

The distribution of abundant MCH containing perikarya in the NLT and some in the NRL of the tilapia hypothalamus, as well as their projections to the pituitary and several brain areas is in line with findings on other teleosts [Naito *et al*, 1985, Batten & Baker, 1988, Bird *et al*, 1989, Baker, 1991] The difference in neuronal size, i e magnocellular MCH containing neurons in the NLT, and smaller cells in the NRL, as was demonstrated here for the tilapia, only has been reported before for the molly *Poecilia latipinna* [Batten & Baker, 1988] In contrast with the immunological staining, ppMCH mRNA was only found in perikarya of the NLT and NRL of the hypothalamus, but not in MCH-immunoreactive fibers in the brain In this report we also explored the possibility to measure ppMCH mRNA levels in the NLT and NRL by separately dissecting these regions out of the brain Using the dot blot assay we showed high levels of ppMCH mRNA in the NLT (hundreds of picograms) and much lower levels in the NRL (tens of picograms) The quantitative distribution fits with the *in situ* hybridization results showing the majority of ppMCH mRNA containing perikarya in the NLT This assay will enable us to investigate differential effects of environmental changes on the two cell populations

In this study we demonstrated the presence of ppMCH mRNA containing cell bodies that were also MCH immunoreactive, in the neurohypophysis of tilapia Although the

anatomical distribution of MCH has been studied in a number of other teleosts and in some mammalian species, the occurrence of MCH-cell bodies in the pituitary has never been reported before [Baker, 1991] In elasmobranchs MCH-containing cells were localized, by immunocytochemistry, in the caudal part of the median lobe of the pars distalis of the pituitary However, this immunoreactivity was not located in the neural tissue of the lobe [Vallarino *et al*, 1989] Tilapia ppMCH mRNA was never detected in axons in the neurohypophysis, where MCH immunostaining is very abundant, which is in accordance with the rule that axons lack mRNA [Gordon-Weeks, 1988]

It remains to be established, what type of cell in the tilapia neurohypophysis contains MCH and what its biological function is The only cell bodies that have been reported for the neurohypophysis teleosts are pituicytes, a kind of specialized astrocytes [Holmes & Ball, 1974] Perhaps, tilapia neurohypophysial ppMCH mRNA is produced in - a subpopulation of - these pituicytes In rats, Schafer and coworkers detected mRNA of the neuropeptide enkephalin in pituicytes of the neural lobe by *in situ* hybridization [Schafer *et al*, 1990] However, pro-enkephalin mRNA-containing cells seem to be more widely distributed through the rat neurohypophysis than MCH-synthesizing cells in the neurohypophysis of tilapia Nevertheless, we investigated the possibility that neurohypophysial ppMCH mRNA is produced in pituicytes We incubated pituitary sections with antiserum to mammalian glial fibrillary acidic protein, a known marker for pituicytes [Salm *et al*, 1982], but we were unable to stain pituicytes in tilapia with this antiserum Hence, at this stage there is no evidence that the MCH-producing cells in the neurohypophysis are pituicytes Another explanation for the presence of MCH producing cells in the neurohypophysis is that they represent perikarya of hypothalamic origin that migrated along axonal tracks to the pituitary Migration of neurons along axonal tracks has been described before for invertebates [Wendelaar Bonga, 1970], but never for vertebrates An observation supporting this explanation is that the few MCH-containing cells present in the pituitary usually are located in the dorsal part of the neurohypophysis, and less frequently in the parts penetrating deeper into the intermediate lobe The function of these neurohypophysial MCH cells is yet unclear They might have a neuro-endocrine function, although in that case, quantitatively, their contribution to the MCH release into the blood seems to be limited, since overall the MCH immunoreactivity in the neurohypophysial MCH cells is much lower than in the surrounding axons derived from the NLT, and the level of ppMCH mRNA in the NIL is very low if compared with the level in the NLT Alternatively, the neurohypophysial MCH cells might have a local regulatory function



Chapter 4

Differential melanin-concentrating hormone gene expression in two hypothalamic nuclei of the teleost tilapia in response to environmental changes

ABSTRACT

For some teleosts, a role has been established for melanin-concentrating hormone (MCH) background adaptation and stress response In teleost fishes, prepro-MCH (ppMCH) mRNA is expressed in the hypothalamus, predominantly in neurons of the nucleus lateralis tuberis (NLT) and in scattered cells of the nucleus recessus lateralis (NRL) The response of mature tilapia to different environmental challenges was studied by assessing ppMCH mRNA levels in these two hypothalamic nuclei by quantitative dot blot analysis Changes in background colour induced pronounced differences in ppMCH mRNA expression in the NLT, but not in the NRL The NLT of tilapia adapted to a white background contained 2 5 to 3 times more ppMCH mRNA than the NLT of black-adapted fish The NLT of fish kept on neutral background contained intermediate levels of ppMCH mRNA, which were significantly lower than the levels in white-adapted fish Oral administration of dexamethasone lowered plasma cortisol concentrations, but had no effect on ppMCH mRNA levels in white- and black adapted fish In tilapia exposed to strongly acidified water (pH 3 5), plasma cortisol and ACTH concentrations were highly elevated, and plasma chloride concentrations considerably lower than in controls These fish responded with a 70 % rise in ppMCH mRNA levels in the NLT, which is most probably associated with a stress response evoked by inadequate osmoregulation After exposure to a milder acidification (pH 4 0) or to seawater no significant changes in ppMCH mRNA levels occurred in either the NLT or the NRL, nor in plasma chloride, cortisol and ACTH levels A specific increase of ppMCH mRNA levels in the NRL was observed in repeatedly disturbed tilapia We conclude that MCH neurons in the NLT and NRL of this teleost differentially respond to background colour, acidification and disturbance, and that this response is not strictly associated with changes in plasma ions and activity of the pituitary-interrenal axis

With P H M Balm G J M Martens & S E Wendelaar Bonga
Journal of Neuroendocrinology (In press)

INTRODUCTION

Melanin concentrating hormone (MCH) is a vertebrate neuropeptide, which is predominantly synthesized in the hypothalamus MCH was first discovered in teleost fish as a neurohypophysial hormone which controls the skin colour [Baker, 1991] More recently, the occurrence of MCH and its preprohormone, ppMCH, were demonstrated in several non-teleostean vertebrate species by immunocytochemistry and molecular cloning [Vaughan *et al*, 1989, Nahon *et al*, 1989, Presse *et al*, 1990, Breton *et al*, 1993a] Subsequently, a number of other functions have been attributed to MCH and to a second peptide derived from ppMCH, called neuropeptide EI (NEI) in mammals and MCH gene-related peptide (Mgrp) in teleosts [Baker, 1991] Recent studies on mammalian MCH, NEI and MCH gene expression showed responsiveness of MCH cells to conditions, such as stress [Baker, 1994], osmotic stimuli [Presse *et al*, 1992, 1993, Fellmann *et al*, 1993, Nahon *et al*, 1993], and lactation [Zamir *et al*, 1986, Bittencourt *et al*, 1992, Knollema *et al*, 1992, Parkes & Vale, 1993]

In teleosts, in addition to the melanin-concentrating function, a role for MCH in the stress-response has been postulated [Baker, 1991] Plasma MCH concentrations increased in trout stressed by injection, and this increase was antagonized by the synthetic corticosteroid dexamethasone, indicating a negative feedback control of cortisol on MCH secretion [Green & Baker, 1991] Lowering the water level and injection stress also influenced MCH biosynthesis in trout [Baker & Bird, 1992] MCH appeared to have modulatory actions on the activity of the hypothalamus-pituitary-interrenal (HPI) axis at several levels MCH inhibits the release of corticotropin releasing factor (CRF) from the hypothalamus and ACTH release from the pituitary gland *in vitro* [Baker *et al*, 1985, 1986, Baker, 1991] MCH also inhibits the release of α-MSH from the pituitary of some teleosts [Barber *et al*, 1987, Balm *et al*, 1993], in fish α-MSH seems to function in background adaptation as well as in the stress response [Lamers *et al*, 1992, 1994, Balm *et al*, 1995]

Osmoregulation and stress responses are intimately interrelated in fish [Eddy, 1981], yet a role for MCH in hydromineral regulation has not been reported in teleosts Therefore, in the present study the response at the mRNA level of MCH neurons to osmoregulatory challenges was investigated in the cichlid teleost *Oreochromis mossambicus*, a tilapia with impressive adaptive capacities, in particular with respect to osmoregulation [Wendelaar Bonga & Balm, 1989] In this fish [Groneveld *et al*, 1995a], as in other teleosts [Baker, 1991], MCH is synthesized in two nuclei of the hypothalamus An abundance of magnocellular MCH neurons has been localized ventrally in the nucleus lateralis tuberis (NLT) Most of these neurons project into the pituitary Scattered MCH cell bodies can be found near the lateral ventricular recess in the nucleus recessus lateralis (NRL) Axonal projections of these neurons have as yet not been identified [Baker, 1991] We recently cloned a hypothalamic ppMCH cDNA of

tilapia [Groneveld *et al*, 1993], and developed a method to measure ppMCH mRNA levels of the NLT and NRL in individual animals [Groneveld *et al*, 1995a] This enabled us to study whether these MCH-synthesizing neurons differentially respond to environmental challenges Since MCH appears to be primarily involved in long-term adaptation processes [Baker, 1991, Baker & Bird, 1992], an inventory was made of MCH gene expression in these two hypothalamic nuclei after a prolonged period of exposure to different background colours, dexamethasone treatment, and exposure to disturbance In addition, levels of ppMCH mRNA were quantified after long-term exposure to seawater and low pH, treatments associated with hydromineral imbalance and endocrine responses [Evans, 1979, Wendelaar Bonga & Balm, 1989], which have been extensively characterized before in our laboratory [Balm, 1986, Wendelaar Bonga & Balm, 1989] As parameters for stress and hydromineral acclimation, plasma cortisol, ACTH and chloride concentrations were determined

MATERIALS AND METHODS

Fish

Tilapia were bred in the laboratory and fed twice daily, each time 1 % of BW, commercial dried fish food (Tetramin) The fish were held at 26 °C in continuously aerated and filtered freshwater under a 12 h light, 12 h dark cycle Sexually mature tilapia, both males and females were used After the experiments the animals were netted and directly before sacrifice blood was collected from the caudal vessels in EDTA/aprotinin (1 5 mg Na_2EDTA, 3000 KIU aprotinin (Serva) ml^{-1}) and centrifuged at 4 °C The plasma was stored at -20 °C until assay After sacrifice by spinal transection, the hypothalamus was dissected from the brain The NLT and NRL were separated by a transverse incision from the ventral side of the hypothalamus, just caudal of the pituitary to the dorsal side of the optic chiasm [Groneveld *et al*, 1995a]

Background adaptation

Male tilapia (BW 17 7 ± 0 5 g) were adapted to white or black backgrounds by transferring them to plastic white or black tanks, respectively, all-glass aquaria on a grey ground in which the stocks are kept were designated as neutral background The tanks contained 80 l of Nijmegen tap water After two weeks the fish were sacrificed

Dexamethasone treatment

Male tilapia (BW 20 0 ± 0 8 g) were adapted to black and white backgrounds as described above Four groups (2 black, 2 white) of 7 fishes were kept for 31 days in the tanks The last

four days before sacrifice one black and one white group of tilapia were fed twice daily with Tetramin flakes (1% of body weight per meal) containing 0 15 mg/g dexamethasone These flakes were prepared by spraying with dexamethasone dissolved in ethanol, which was allowed to evaporate overnight at room temperature Control groups received the same quantity of Tetramin flakes sprayed with ethanol only At day 31, 16 h after the last feeding, the fish were captured

Exposure to acidified and saltwater

Three groups of male tilapia (BW 20 5 ± 0 5 g) were kept 7 days before the start of the experiments on a neutral background in 120 l glass aquaria containing artificial freshwater, consisting of demineralized water supplemented with 1 3 mM $NaHCO_3$, 0 5 mM $CaCl_2$, 0 06 mM KCl and 0 2 mM $MgCl_2$ at pH 7 8 [Balm, 1986] For exposure to acidified water the pH was lowered gradually to pH 3 5 over a period of 75 h by addition of H_2SO_4 via a flow through system For exposure to 70 % seawater the water osmolarity was raised gradually from 5 mosm/kg to 700 mosm/kg over a period of 48 h by addition of seawater (artificial fresh water salted with Wimex seasalts, Wieland & Co, Krefeld, Germany) via a flow-through system The water pH and osmolarity were controlled without disturbing the fish by taking water samples from the effluent outside the climate chamber The fish were kept at low pH or in seawater for 10 days Control fish were kept in artificial freshwater Plasma chloride concentrations were measured by flame photometry as parameter for osmotic adaptation

Exposure to disturbance stressors

Six weeks before the start of the experiments female tilapia (BW 14 8 ± 1 2 g) were divided into 2 groups, and kept on a neutral background in 80 l glass aquaria with continuously filtered and refreshed artificial freshwater One group was exposed to disturbance stressors during 6 days This group was housed in a separate identical climate room The fish of this group were daily disturbed by confining them in a small net, switching off the light, and switching off the oxygen supply, each for 10 min in random order, and at irregular intervals to prevent habituation Feeding and disturbance ended one day before sacrifice

RIA for cortisol

Plasma concentrations of cortisol were determined in a RIA for cortisol as described previously [Balm *et al*, 1994] Plasma samples were diluted 10 times with distilled water before assay The cortisol antiserum was purchased from Steranti Res Ltd (U K) Cross reactivity with dexamethasone was determined to be 3 % The antiserum was diluted to yield one third of the titer recommended by the suppliers ^{3}H labelled cortisol was from Amersham

Free and bound cortisol were separated by precipitation of the immunocomplex with dextran-coated charcoal The inter-assay variation was 8 5 % and the intra-assay variation was 7 9 %

RIA for ACTH

Plasma concentrations of ACTH were determined in a RIA for ACTH as described previously [Balm *et al* , 1994] The antiserum against $ACTH_{1\ 24}$ was kindly provided by Prof R Dores, University of Denver, USA The final dilution was 1 38,500 The $hACTH_{1\ 39}$ standard was from NIBSS (London, U K) Iodinated ACTH was from Amersham Free and bound ACTH were separated by precipitation with PEG 6000 (Merck) after incubation with a second sheep anti rabbit IgG antibody The inter-assay variation was 7 2 %, and the intra-assay variation was 6 2 %

Measurements of ppMCH mRNA

Levels of ppMCH mRNA in the NLT and NRL were measured by dot blot analysis as described previously [Groneveld *et al* , 1995a] In short, total RNA was isolated from separated hypothalamic NLT and NRL regions by the acid guanidinium-thiocyanate phenol chloroform procedure [Chomczynski & Sacchi, 1987], yielding about 3 µg total RNA per NLT and about 8 µg per NRL, in accordance with the size of the tissue samples RNA was resuspended in 5 x SSPE (1 x SSPE is 0 18 M NaCl, 0 01 M NaH_2PO_4, pH 7 4, 0 001 M EDTA), 7 4 % formaldehyde solution, and 25 or 33 % of the NLT RNA and 100 % of the NRL RNA was blotted on nitrocellulose using a dot blot apparatus (Bio Rad) Dot blots containing a sense tilapia proMCH cRNA standard dilution series were hybridized with a ^{32}P labelled antisense cRNA probe derived from clone TMe58, containing a 270-bp tilapia ppMCH cDNA fragment, encoding tilapia Mgrp and MCH and part of the 3' untranslated region [Groneveld *et al* , 1993] Washing was performed until 0 1 x SSPE, 0 1 % SDS, 68 °C Hybridization signals were quantified by densitometric scanning of the autoradiograms The values were converted by the sense proMCH cRNA standard curve to pg ppMCH mRNA/g BW The detection limit is 1 pg of ppMCH mRNA/dot

Statistical analysis

Data are presented as means ± S E M For statistical analysis the two-tailed Student's *t* test was used after log transformation of data Significance was accepted at $P < 0\ 05$

RESULTS

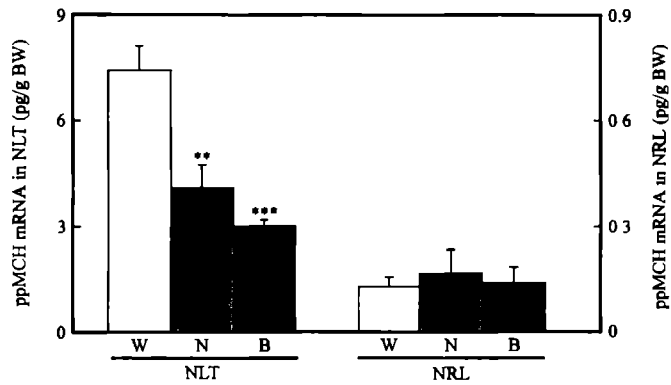


Figure 4.1 Effect of background colour on ppMCH mRNA expression in tilapia NLT and NRL Total RNA extracted from the NLT and NRL of tilapia adapted for two weeks to either a white, neutral or black background, was dot blotted and hybridized with a tilapia-specific anti-sense MCH cRNA probe. A sense MCH cRNA standard curve was used to convert hybridization signals into picograms of ppMCH mRNA per gram body weight. W, white-adapted tilapia (n = 7), N, tilapia adapted to neutral background (glass aquaria; n = 5); B, black-adapted animals (n = 9) ** $P < 0.01$, *** $P < 0.001$ compared with the NLT of W

Effect of background colour and dexamethasone on tilapia ppMCH mRNA expression

Tilapia kept up to 1 month in white or black tanks showed a pale or black skin-colour, respectively. As shown in Fig. 4.1, in the NLT of tilapia adapted for two weeks to a white background the ppMCH mRNA level was 2.5 times higher than the NLT of black-adapted fish, whereas the NLT of fish adapted to a neutral background contained intermediate levels of ppMCH mRNA, which were significantly lower than the levels found in white-adapted fish. No differences were found in the NRL region. Similar results were obtained when tilapia were adapted to black and white backgrounds for one month (Fig. 4.2). Stress-free, oral dexamethasone administration to white- and black-adapted tilapia significantly decreased plasma cortisol concentrations in both groups, but did not change plasma ACTH levels (Table 4.1). The dexamethasone treatment had no effect on ppMCH mRNA levels in either the NLT or NRL of white- and black-adapted tilapia (Fig. 4.2).

Effect of osmotic challenges and disturbance on tilapia ppMCH mRNA expression

In the following experiments fish were kept on a neutral background and no change of skin colour was observed. In tilapia, exposed to acidified water of pH 3.5, plasma cortisol and

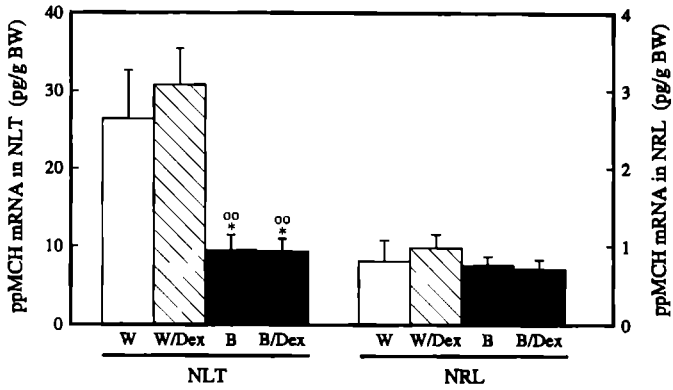


Figure 4.2 Effects of dexamethasone and background colour on ppMCH mRNA expression in tilapia NLT and NRL ppMCH mRNA expression levels per gram body weight are shown W, white-adapted tilapia, W/Dex, white-adapted dexamethasone treated tilapia, B, black-adapted animals, B/Dex, black-adapted dexamethasone treated fish n = 7 * $P < 0.05$ compared with the NLT of white-adapted tilapia, oo $P < 0.01$ compared with the NLT of white-adapted, dexamethasone treated animals.

Table 4.1 Effect of dexamethasone administration and background colour on the concentration of cortisol and ACTH in tilapia plasma

Treatment	**Cortisol** (ng/ml)	**ACTH** (pg/ml)
White-adapted		
Control	148.4 ± 20.2	21.9 ± 5.0
Dexamethasone	32.6 ± 4.4***	25.1 ± 1.5
Black-adapted		
Control	128.4 ± 27.3	18.8 ± 2.9
Dexamethasone	31.7 ± 4.5**	24.5 ± 3.0

Fish were maintained in either white or black tanks for one month. The last 4 days they were fed daily 2 % of bodyweight untreated Tetramin flakes (control), or 0.15 mg/g dexamethasone treated flakes The fish were killed 16 h after the last feeding Values are means ± S E M (n=7) ** $P < 0.01$, *** $P < 0.001$ compared with control fish

ACTH levels were strongly elevated compared with controls from pH 7.8 freshwater, while plasma chloride concentrations decreased by 35 % (Table 4.2) These fish most of the time stayed on the bottom of the aquarium and did not react quickly to food Levels of ppMCH

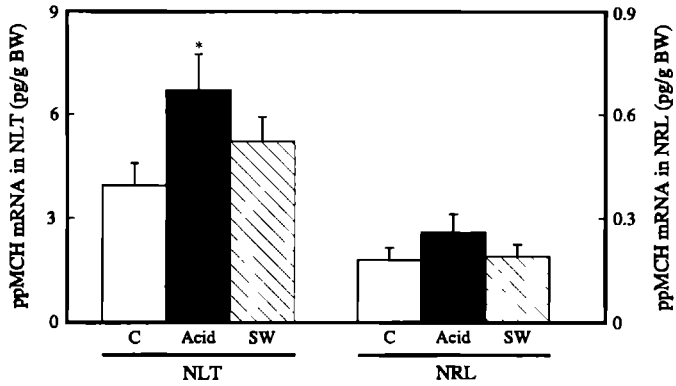


Figure 4.3 Effect of osmotic challenges on ppMCH mRNA expression in tilapia NLT and NRL. ppMCH mRNA expression levels per gram body weight are shown for pH 7.8 freshwater tilapia (C), pH 3.5 freshwater animals (Acid) and 70 % seawater exposed fish (SW). n = 8. * $P < 0.05$ vs the NLT of control fish.

Table 4.2 Effect of acidified and seawater exposure on the concentration of cortisol and ACTH, and chloride in tilapia plasma.

Treatment	Cortisol (ng/ml)	ACTH (pg/ml)	Cl^- (mM)
Control	136.0 ± 28.2	39.8 ± 3.5	149.1 ± 2.2
pH 3.5	501.5 ± 84.9**	214.8 ± 37.3***	96.8 ± 3.6***
70 % seawater	203.8 ± 41.7	61.0 ± 8.5	149.6 ± 2.2

Fish were maintained on neutral background in freshwater of either pH 7.8 (control), or pH 3.5, or in 70 % seawater for 10 days. Values are means ± S.E.M., n = 8. ** $P < 0.01$, *** $P < 0.001$ compared with control fish.

mRNA in the NLT of the pH 3.5-exposed animals increased by 70 % above controls, while no statistically significant changes could be measured in the NRL (Fig. 4.3). In a subsequent experiment, tilapia exposed to water of pH 4.0 showed no signs of discomfort, and no significant changes were measured in either plasma cortisol, ACTH, chloride (data not shown), or in hypothalamic ppMCH mRNA concentrations (NLT, C: 9.86 ± 0.79 pg/g body weight [BW]; pH 4.0: 12.57 ± 1.51 pg/ g BW; NRL, C: 0.77 ± 0.09 pg/g BW; pH 4.0: 0.82 ± 0.10 pg/g BW). Tilapia exposed for 10 days to 70 % seawater showed no statistically significant

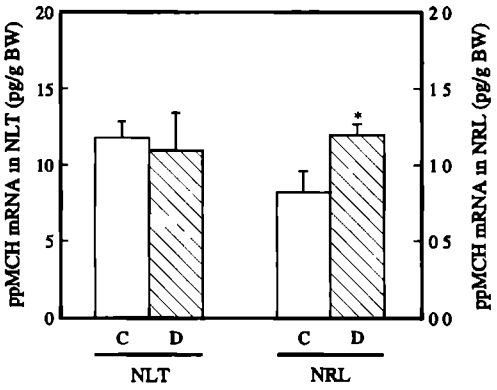


Figure 4.4 Effect of disturbance on ppMCH mRNA expression in NLT and NRL of tilapia ppMCH mRNA expression levels per gram body weight are shown for freshwater controls (C, n = 4) and disturbance treated fish (D, n = 6) * $P < 0.05$, vs the NRL of controls

differences in hypothalamic ppMCH mRNA levels, nor in plasma cortisol, ACTH or chloride concentrations when compared with freshwater controls (Fig 4.3, Table 4.2)

Tilapia disturbed three times per day at irregular intervals, showed a 45 % increase in ppMCH mRNA levels in the NRL when compared with control levels (Fig 4.4) No changes in ppMCH mRNA levels were found in the NLT in response to this treatment

DISCUSSION

The present study provides evidence that hypothalamic MCH-neurons in the NLT and NRL differentially respond to environmental challenges NLT neurons respond to changes in background colouration and acidification of the water, while MCH-neurons of the NRL respond to repeated disturbance

The levels of ppMCH mRNA expression in the two hypothalamic nuclei of controls varied between different experiments We consider this to be due to a reasonable range of biological variation, most probably related to the batch of fish Within one experiment tilapia from one batch of eggs were used, but in the set of experiments described different batches were used Moreover, in a series of background adaptation experiments, it was our experience that although absolute ppMCH mRNA values sometimes differ a factor two to four between experiments (compare for example Figs 4.1 and 4.2) the difference between white- and black-adapted fish was always a factor three

A prominent rise in ppMCH mRNA expression levels was found in the NLT but not in the NRL when tilapia were kept on a white background instead of a black or neutral

background We conclude from our refined approach, to study the two hypothalamic regions separately, that the rise of tilapia hypothalamic ppMCH mRNA levels that we have previously reported [Groneveld *et al*, 1995a] resulted specifically from a rise in MCH neurons of the NLT Consistent with the enhancement of hypothalamic ppMCH MRNA levels, Baker and Bird [1992] reported that *de novo* MCH synthesis in whole hypothalami of trout on a white background is doubled when compared with that of black-adapted animals The only report specifically dealing with changes in the NLT, but not referring to neurons of the NRL, concerns a morphological study in Chinese grass carp indicating that MCH neurons are more active in white-adapted fish the cells had larger cytoplasmic and nuclear areas and more prominent nucleoli than those of black-adapted animals [Bird & Baker, 1989] The specific rise of ppMCH mRNA levels in neurons of the NLT, projecting mainly to the neurohypophysis [Baker, 1991], is in accordance with the reported increase of MCH secretion from the pituitary in white-adapted teleosts In white-adapted trout, more MCH is present in the blood than in black-adapted fish [Baker, 1991] Also in eel and carp the rate of MCH secretion differs in response to changes in background colouration, as judged from the MCH content of the pituitary gland [Baker, 1991]

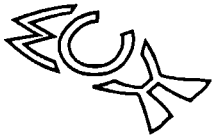
As the neurons of the NRL were apparently not responding to changes in background colouration, we searched for other functions Since in trout MCH appears to be involved in the modulation of the hypothalamus-pituitary-interrenal (HPI) axis [Baker *et al*, 1985, 1986, Baker, 1991, Green & Baker, 1991, Baker & Bird, 1992], we followed several approaches to manipulate the HPI axis activity We reduced the output of the interrenal tissue by feeding dexamethasone to white- and black adapted tilapia This treatment lowered plasma cortisol levels, which is in line with similar observations on brown trout at neutral background [Pickering *et al*, 198] Plasma ACTH levels were not altered, which corresponds with findings after *in vivo* cortisol administration to this tilapia [Balm *et al*, 1994] The dexamethasone treatment did no alter ppMCH mRNA levels of both hypothalamic MCH neuron groups of tilapia kept on black as well as white backgrounds, which corroborates findings in trout In these fish injection of dexamethasone suppressed the stress-induced rise in plasma MCH levels as effectively as it suppressed the stress-associated rise in plasma cortisol, but it did not significantly influence background colour-related plasma MCH levels [Green & Baker, 1991] Although to our fish a stress-free dexamethasone treatment was given, they were stressed by the capture procedure Tilapia responds extremely fast to capture with a surge in plasma cortisol levels, which is ACTH-independent, but subject to feedback by cortisol [Balm *et al*, 1994] and dexamethasone (this study) This sampling effect occurs within minutes, whereas in the study by Green and Baker [1991] fish received daily injections, and the experiment was terminated one hour after the final injection It is conceivable that in our case the sampling period was too short for the MCH neurons to react It is also possible that only MCH release,

but not MCH biosynthesis, is influenced by dexamethasone, since dexamethasone treatment had no significant effect on levels of newly synthesized trout ppMCH [Baker & Bird, 1992]

We then exposed the fish to strongly acidified water (pH 3 5), a treatment which is known to evoke a prominent response of the HPI axis Wendelaar Bonga & Balm, 1989] In response to acidified water elevated plasma levels of cortisol, acting as a mineralocorticoid, are known to counteract the disturbed ionoregulation [Wendelaar Bonga & Balm, 1989] Indications that exposure to water of pH 3 5 was experienced as a severe stressor by our fish are the extremely high plasma cortisol and ACTH values and the apparent discomfort of these animals This challenge significantly increased ppMCH mRNA levels of the NLT, but not of the NRL This stimulation of MCH neurons of the NLT is most probably associated with a stress response evoked by inadequate osmoregulation in these fish, evidenced by the above mentioned findings Exposure to pH 4 0, or to 70 % seawater did not alter ppMCH mRNA levels, which may indicate that the response of MCH neurons to challenges affecting osmoregulation is limited to conditions to which the fish do not acclimate, and in which the hydromineral balance is notably disturbed Acclimation of tilapia to pH 4 0 and to seawater was apparent from the unchanged plasma cortisol and chloride values, consistent with results described before [Evans, 1979, Balm, 1986, Wendelaar Bonga & Balm, 1989]

As a third type of stressor, tilapia were exposed to repeated disturbance This treatment, which has no effect on plasma chloride and cortisol levels (Pelgrom, Balm personal communication), induced a significant increase of ppMCH mRNA expression in the NRL, whereas ppMCH mRNA levels of the NLT appeared to be unaffected Thus MCH neurons in the NRL specifically respond to this disturbance, while they are not responsive to background colouration or osmotic challenges Altogether, the above findings demonstrate that ppMCH mRNA expression is differentially regulated in the NLT and NRL of tilapia, which suggests that these two MCH-synthesizing nuclei are part of different and stressor-specific pathways

Our results confirm for tilapia the complex relationship between MCH and stress response, described for trout by Baker and coworkers [Baker, 1991, Green & Baker, 1991, Baker & Bird, 1992] We found that after exposure of tilapia to acidification, ppMCH mRNA levels in the NLT and plasma cortisol concentrations were positively related However, in the experiments dealing with background adaptation or dexamethasone no such relationship was found In addition, no relationship was found between plasma cortisol values and ppMCH mRNA levels in the NRL of tilapia Therefore, we conclude that stimulation or inhibition of MCH synthetic activity is not strictly coupled to the interrenal stress response



Chapter 5

Biphasic effect of MCH on α-MSH release from the tilapia (*Oreochromis mossambicus*) pituitary

ABSTRACT

The effect of melanin-concentrating hormone (MCH) on the release of α-melanocyte-stimulating hormone (α-MSH) from the tilapia pituitary gland was studied *in vitro* In a superfusion set up, 10 nM to 1 μM synthetic salmon MCH caused a concentration dependent inhibition of α-MSH release from tilapia neurointermediate lobes (NILs) Immunoneutralization of MCH in tilapia NILs further indicated that endogenous MCH has an inhibitory effect on the melanotropes The release of mono-acetylated α-MSH release was more strongly inhibited by MCH than that of des-, and di-acetylated α-MSH, indicating that MCH modulates the secretory signal of the melanotropes in a quantitative and qualitative manner A high concentration of MCH (10 μM) substantially increased the release of α-MSH Further evidence in support of a stimulatory action of high concentrations of MCH was provided by the observation that the MCH analog $MCH_{2\ 17}$ at 10 and 35 μM enhanced α-MSH release as well Therefore, we conclude that the response of pituitary melanotropes to MCH is biphasic, as was reported before for the effects of MCH on other targets in fish and mammals Under physiological conditions the inhibitory action of MCH on fish melanotropes most likely dominates

INTRODUCTION

Many lower vertebrates alter the colour of their skin in response to variations in background colouration Background adaptation is of paramount importance to these animals for survival. The regulation of background adaptation represents an attractive

With P H M Balm & S E Wendelaar Bonga
Peptides 16 XX-XX (1995)

model for the analysis of the interactions between peptidergic neuroendocrine factors In most species α-melanocyte-stimulating hormone (α-MSH), dominates the hormonal control of pigment migration in response to changes in background coloration α-MSH is produced in the melanotropes of the pars intermedia of most vertebrates, and induces pigment dispersion [Bagnara & Hadley, 1973] In teleosts a neurohormone, melanin-concentrating hormone (MCH), antagonizes α-MSH in this respect [Baker & Ball, 1975] As in other teleosts [Baker, 1991], in tilapia (*Oreochromis mossambicus*) MCH is synthesized in the hypothalamus and transported to the pituitary pars nervosa [Groneveld *et al*, 1993] Also in higher vertebrates, MCH is produced in the hypothalamus [Baker, 1991], and it plays a role in the stress response, in osmoregulation, and in lactation [Baker, 1994] In teleosts the MCH system appeared to be responsive to stress and ionic challenges as well [Baker, 1991, Green & Baker, 1991, Green *et al*, 1991, Baker & Bird, 1992, Groneveld *et al*, 1995b], in addition to its function in the regulation of skin colour

There are indications that MCH may affect background adaptation at the level of the pituitary by inhibiting α-MSH release from the pars intermedia Firstly, MCH nerve terminals are located in the vicinity of pars intermedia cells [Batten & Baker, 1988, Powel & Baker, 1988] Furthermore, it has been reported that trout injected with MCH had decreased plasma α MSH levels [Baker & Bird, 1986], and finally immunoabsorbance of endogenous MCH in trout and eel pituitary tissue enhanced the release of α-MSH *in vitro* [Barber *et al*, 1987]

At present, little is known on the sensitivity of pituitary melanotropes to MCH, and on species differences for the effect of MCH on melanotropes In the teleost skin, MCH induces aggregation of melanin at concentrations that occur in the blood [Baker, 1991, 1993], whereas administration of high concentrations of MCH to melanophores induces pigment dispersion The latter has been explained by binding of MCH to α-MSH-receptors on the melanophores [Castrucci *et al*, 1987, Hadley *et al*, 1988] The MCH concentration-response curve of MCH on auditory gating in rats is also biphasic [Miller *et al*, 1993] This raises the question whether the concentration response relationship of MCH on α-MSH release is biphasic as well

Three forms of α-MSH are known des-, mono-, and di-acetylated α-MSH In tilapia, all three α-MSH forms are released from the neurointermediate lobe (NIL) [Lamers *et al*, 1991] Acetylation of α MSH is of functional significance, since the isoforms of α-MSH have different melanotropic and corticotropic potencies in fish [Kishida *et al*, 1988, Lamers *et al*, 1992] Mono-acetylated and di-acetylated α-MSH are most effective on pigment dispersion in trout [Kishida *et al*, 1988], whereas in particular di-acetylated α-MSH has corticotropic activity in tilapia [Lamers *et al*, 1992, Balm *et al*, 1995]

The purpose of the present study was to gain more insight into the action of MCH at the pituitary level We therefore quantitatively and qualitatively analyzed the effects of MCH and $MCH_{2\ 17}$, a MCH analog lacking the N-terminal amino acid, which was helpful in elucidating the mechanisms of action of MCH on the teleost skin [Castrucci *et al*, 1989, Matsunaga *et al*, 1989] on the release of α-MSH *in vitro* from the tilapia pituitary gland

MATERIALS & METHODS

Animals

Male and female tilapia (*Oreochromis mossambicus*), average weight 80 g, were bred in the aquarium facility of the Dept of Animal Physiology of the University of Nijmegen They were kept in fresh-water at 28°C and were fed a commercial dried fish food (Tetramin) The fish were kept in glass aquaria, illuminated by overhead TL tubes, with a day-night rhythm of 12 h light and 12 h darkness Immediately after removal from the tank, the animals were sacrificed by spinal transection and pituitary glands were dissected from the brain

Superfusion of neurointermediate lobe tissue

One or two freshly dissected pituitaries or NILs were placed on a nylon gauze in a 10 µl superfusion chamber A multichannel peristaltic pump (Whatson Marlow) was used to pump carbogen aerated incubation medium (IM) (142 mM NaCl, 2mM KCl, 2 mM $CaCl_2$, 15 mM HEPES (pH 7 38), 0 3 mg/ml bovine serum albumin (Sigma), 2 5 mg/ml glucose) through each chamber at a rate of 30 µl/min The superfusion chambers as well as the IM were thermostat controlled at 28°C The effluent from each chamber was collected in fractions over varying periods with an Isco (Retriever II model) fraction collector During at least 3 h superfusion fractions were collected before switching to 'pulse' medium containing MCH or $MCH_{2\ 17}$ (both synthesized by Dr T O Matsunaga at the lab of Dr V J Hruby and kindly provided by Dr M E Hadley, Tuscon, USA) [Wilkes *et al*, 1984], for a 25 to 30 min period After the pulse the superfusion was continued with IM The collected fractions were stored immediately at -20°C until α-MSH radioimmunoassay

Immunoneutralization of MCH from tilapia NILs

NILs from tilapia kept in dark glass aquaria were dissected and individually pre-incubated in 100 µl IM, containing 5 mg/ml (w/v) glucose, during 1 h at 28°C Subsequently, the NILs were transferred into 30 µl IM containing either 0 1% (v/v) MCH antiserum (a kind

gift of Dr B I Baker and Dr H Kawauchi) [Naito *et al*, 1985] or 0 1% (v/v) normal rabbit serum The vials were aerated with carbogen, sealed, and shaken gently at 28°C for a 15 h incubation period After this static incubation, media were collected and stored at -20 °C until α-MSH radioimmunoassay, subsequently, the NILs were superfused as described above

Reversed phase-HPLC analysis

To determine the effect of MCH on the profile of the secretory signal, superfusate was submitted to HPLC analysis Different forms of α-MSH were separated on a Spherisorb 10 ODS column (Bisschof) as described before [Lamers *et al*, 1991] In short, the primary solvent was buffer B (0 5 M formic acid, 0 14 M pyridine, pH 3 0) and elution was accomplished with a gradient of 1-propanol at a flow rate of 2 ml/min Fractions of 0 6 ml were collected The fractions were dried in a Speedvac concentrator (Savant), diluted into 0 05 N HCl, 50% methanol and submitted to α MSH radioimmunoassay

α-MSH radioimmunoassay

The α-MSH radioimmunoassay (RIA) with L9 α-MSH antiserum has been described before [Lamers *et al*, 1992] The antiserum is equally sensitive to des-, mono and di-acetyl α-MSH Cross-reactivity of the α-MSH antiserum with MCH (preliminary results have been reported before by De Koning *et al*) [1992] and $MCH_{2\ 17}$ was assessed (see Results)

Processing of data and statistics

The results of the superfusions are either expressed as pg α-MSH per min per NIL, or as a percentage of the basal release (=100 %) Basal release was defined as the average α MSH release in 20 to 30 min prior to addition of secretagogue, and basal release values ranged from 30 to 200 pg/min/NIL For the concentration-response relationships of MCH- or $MCH_{2\ 17}$-induced effects, values for maximal inhibition or stimulation of α-MSH release during the secretagogue pulse were expressed as a percentage of the pre-pulse values Correction of α-MSH values for cross-reactivity after a 10 μM MCH pulse was performed as follows proceeding from 0 0032 % cross-reactivity (see Results) we calculated that 10 μM MCH in a 75 μl RIA sample is equivalent to 40 pg immunoreactive α-MSH This value was subtracted from the RIA data of pulse fractions EC_{50} was estimated as described previously [Lamers *et al*, 1994] Results are presented as means +, or ± SEM For statistical analysis data were log transformed where appropriate For the concentration response relationships statistical analyses were performed using the paired Student's *t* test For each concentration, the maximum value during the pulse of each incubation was tested

against its own control value of basal release In other cases the unpaired Student's *t* test was used To test parallelism of dilution curves the computer program PHARM/PCS-version 4 1 [Tallarida & Murray, 1986] was used

RESULTS

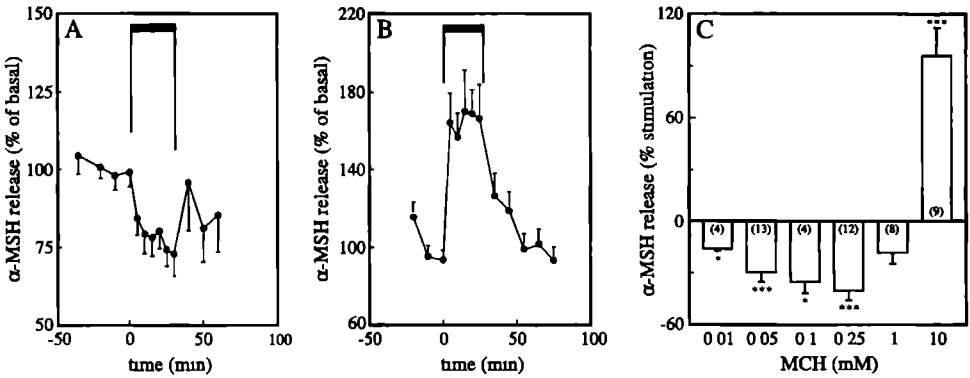


Figure 5.1 Inhibitory (**A**) and stimulatory (**B**) effects of MCH on α-MSH release from tilapia NILs during *in vitro* superfusion expressed as percentage of basal release (= 100%) **A.** 250 nM MCH, n = 12, **B.** 10 μM MCH, n = 9 The time of addition of secretagogue after at least 220 min of superfusion is designated t_0 **C.** Concentration-response relationship of MCH induced effects on α-MSH release Basal release in this presentation is designated 0 % Numbers of incubations are given in parentheses for each concentration tested * $P < 0\,05$, *** $P < 0\,001$

Quantitative effects of MCH on α-MSH release

At concentrations below 1 μM, MCH caused a concentration dependent inhibition of α-MSH release during *in vitro* superfusion of tilapia NILs (Fig 5 1A,C), and at 10 μM α-MSH release was stimulated (Fig 5 1B) The effect of MCH on the α-MSH release lasted for the duration of the pulse, after the pulse α-MSH release did restore to basal levels The MCH concentration inducing 50 % of maximum inhibition of α-MSH release (EC_{50}), using the data points up to and including 250 nM, was estimated to be 19 nM with a maximum inhibition of 42 % At 10 μM MCH the α-MSH release was stimulated with a maximum stimulation of 96 ± 17 % ($P < 0\,001$, Fig 5 1C) Taking into account the slight cross-reactivity of MCH with the α-MSH antiserum (0 0032 %, Fig 5 2), this percentage

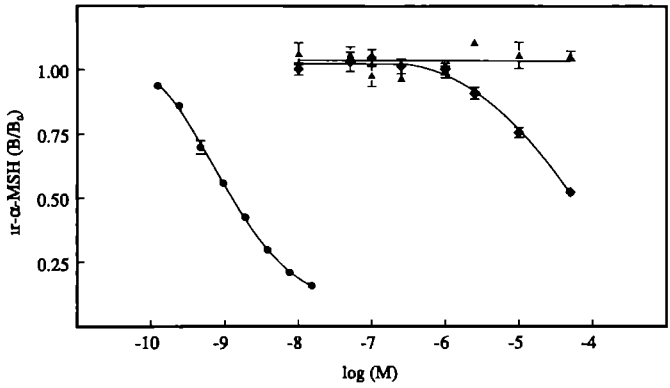


Figure 5.2 Cross-reactivity of MCH with α-MSH antiserum Concentration series of MCH (-♦-) and $MCH_{2\ 17}$ (-▲-) in 50 µl incubation medium were tested for cross-reactivity in α-MSH radioimmunoassay. B/B_0 is compared with the α-MSH standard curve (-●-) Sample size 3 or 4. SEMs not shown fall within the area of the symbol. ED_{50} α-MSH = 1 8 nM, ED_{50} MCH = 57 µM. $MCH_{2\ 17}$ did not cross-react

decreased marginally to 87 ± 22 % ($P < 0.001$ compared with basal release). However, since the dilution curve for MCH only approximately paralleled the α-MSH dilution curve in the α-MSH radioimmunoassay (Fig. 5.2; the slope of log transformed curves was significantly different, $P < 0.01$) an additional control was performed. To mimic pulse conditions, MCH was added to pre-pulse superfusates to a final concentration of 10 µM. This yielded a non-significant increase ($P = 0.88$) of immunoreactive α-MSH of 2 ± 10 pg (and 16 ± 16 %; n = 6) compared with untreated pre-pulse fractions. During the 10 µM MCH pulse the increase of immunoreactive α-MSH was 108 ± 33 pg (95 ± 28 %; n = 5), which was significantly higher ($P < 0.01$) than the increase measured when MCH was added afterwards to superfusates. $MCH_{2\ 17}$, a MCH analog that in contrast to MCH, did not cross-react with the α-MSH antiserum (Fig. 5.2), induced at concentrations of 10 µM and 35 µM a significant stimulation of the α-MSH release (Fig. 5.3). The stimulation of α-MSH release by 10 µM $MCH_{2\ 17}$ was comparable with that evoked by 10 µM MCH.

Effect of endogenous MCH on α-MSH secretion

After incubation of tilapia NILs with 0.1 % MCH antiserum, to immunoneutralize endogenous MCH, α-MSH release significantly increased compared with NILs incubated in medium containing normal rabbit serum (Fig. 5.4A). After 15 h of static incubation, the antiserum was washed out by superfusion of the NILs with fresh incubation medium.

During this superfusion the α-MSH release of the NILs incubated with the MCH antiserum returned to control levels within 2 h (Fig 5 4B)

Effects of MCH on the release of α-MSH isoforms

To study the qualitative effect of MCH on α-MSH release, the isoforms of α-MSH released before and during pulses with 100 nM and 250 nM MCH were analyzed by HPLC Des-, mono-, and di-acetylated α-MSH were released during MCH pulses as well as under control conditions (Fig 5 5) During the 100 nM and 250 nM MCH pulses the amount of mono-acetylated α MSH decreased relatively more than the amounts of des- and di-acetylated α MSH, when compared to control Taking the area under the curve of di-acetylated α-MSH (di), mono-acetylated α-MSH (mono) and des-acetylated α-MSH (des) we calculated di/mono and des/mono ratios of 0 45 and 0 38 for control, 0 73 and 0 50 during a 100 nM MCH pulse, and 0 95 and 0 61 during a 250 nM MCH pulse, respectively The highest increase of the ratios was found at the concentration (250 nM) where the overall inhibitory effect was the strongest (Figs 5 1 and 5 5)

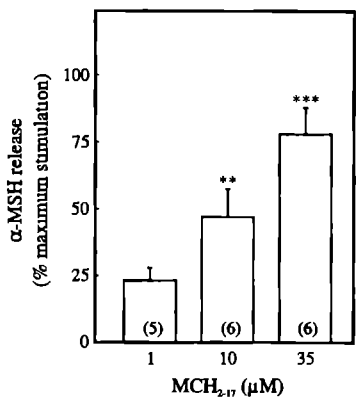


Figure 5.3 Stimulatory effect of $MCH_{2\text{-}17}$ on α-MSH release during *in vitro* superfusion Concentration-response relationship is given for the effect of high concentrations of $MCH_{2\text{-}17}$ on α-MSH release Numbers of incubations are given in parentheses ** $P < 0.01$, *** $P < 0.001$

DISCUSSION

The present results demonstrate that the MCH concentration response curve for α-MSH release from the tilapia NIL is biphasic At concentrations below 1 μM, synthetic MCH caused a concentration dependent inhibition of α MSH release Concentrations above 1 μM of MCH stimulated α-MSH release These results strongly resemble the auto antagonism phenomenon described for the action of MCH on pigment cells in the skin of tropical eel

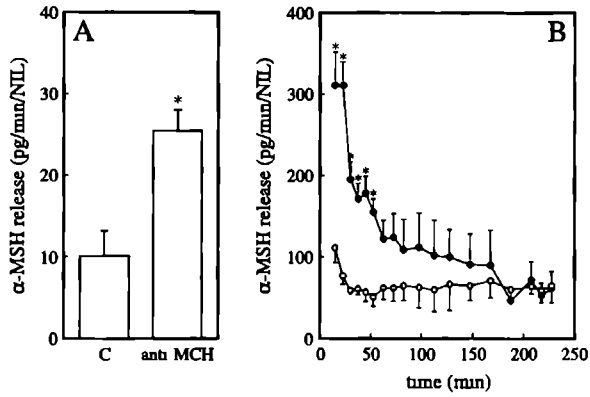


Figure 5.4 Effect of *in vitro* immunoneutralization of endogenously released MCH on α-MSH release from tilapia NILs **A.** α-MSH release of NILs incubated during 15 h with 0 1% MCH antiserum (anti-MCH, n = 4) versus NILs incubated with normal rabbit serum as controls (C, n = 4) **B.** α-MSH release during subsequent superfusion with normal incubation medium of MCH antiserum treated NILs (-●-) and controls (-O-) * $P < 0\ 05$

(*Synbranchus marmoratus*) *in vitro* [Castrucci *et al*, 1987] and the biphasic concentration-response curve of the effect of MCH in auditory gating of rats [Miller *et al*, 1993]

Significant inhibition of α-MSH release from tilapia NILs occurred from 10 to 250 nM MCH In trout, MCH tested at a single dose of 100 nM, did not inhibit α-MSH release *in vitro* [Barber *et al*, 1987] The difference with tilapia could be due to other incubation conditions or to species differences in sensitivity of teleost melanotropes to MCH An attractive hypothesis is that in trout 100 nM MCH reflects the turning-point in the concentration-response curve Tilapia melanotropes appeared to be less sensitive to MCH than tilapia scale melanophores the EC_{50} of the effect of MCH on α-MSH release was 19 nM (this study), whereas the EC_{50} on pigment migration was 1 nM [Kawazoe *et al*, 1987] We reason that the lower sensitivity of melanotropes to MCH reflects the mode of MCH targeting, which is neuroendocrine in the pituitary and in the classical sense endocrine for the skin Pituitary melanotropes are in close contact with the MCH-containing neurons of the neuro-hypophysis of tilapia (personal observation) and other teleosts [Batten & Baker, 1988, Powel & Baker, 1988], and MCH is endogenously released [Barber *et al*, 1987, this study], whereas the effect of MCH on melanophores is exerted by relatively low circulating MCH levels [Eberle *et al*, 1989, Kishida *et al*, 1989] The difference in sensitivity of the tissues can not be attributed to differences in the peptide sequences of synthetic salmon MCH and tilapia MCH, since we previously demonstrated that the sequences of both peptides are identical [Groneveld *et al*, 1993] The sensitivity of tilapia

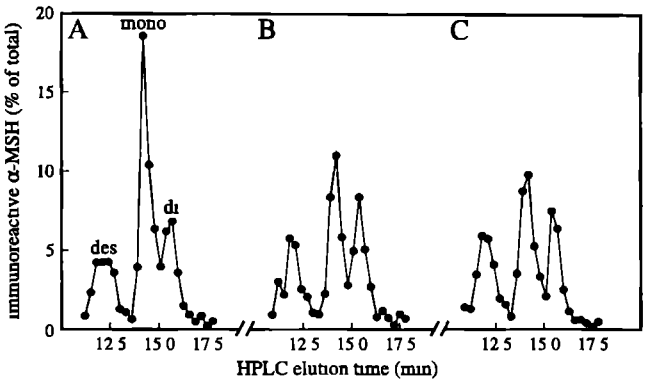


Figure 5.5 HPLC immunogram of α-MSH in pooled NIL superfusion fractions with or without MCH pulse **A.** Control, **B.** 100 nM MCH, maximum inhibition of total α-MSH release in superfusion was 35 ± 7 % (n = 8) **C.** 250 nM MCH maximum inhibition of total α-MSH was 47 ± 6 % (n = 4) Peak identification was based on Lamers *et al* [1991], des = des-acetylated α MSH mono = mono acetylated α-MSH, di = di-acetylated α-MSH

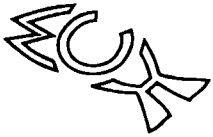
melanotropes to MCH is comparable with the sensitivity to dopamine, another potent inhibitor of α-MSH release in tilapia (EC_{50}, 10 nM, Dr A E Lamers personal communication, see also 25) However, MCH modulates the secretory output of the melanotropes not only quantitatively but also qualitatively by selectively decreasing the release of mono acetylated α-MSH In this respect MCH differs from dopamine, which indiscriminately inhibited the release of mono- and di-acetylated α-MSH from tilapia NILs [Lamers *et al*, 1991] The different modes of action of these two inhibitors might enhance the flexibility of the fish to respond to environmental challenges Tilapia melanotropes are more sensitive to MCH than to thyrotropin-releasing hormone (TRH), a stimulator of α-MSH release (EC_{50}, 200 nM) [Lamers *et al*, 1994] However, with respect to the effect on differential α-MSH release MCH resembles TRH, which also enhances the di/mono ratio of released α-MSH [Lamers *et al*, 1991]

Immunoneutralization of MCH in NILs *in vitro* showed that in tilapia endogenous MCH inhibits α-MSH release from the melanotropes and that this inhibitory effect persists *in vitro* as long as 18 hours, since after wash-out of the MCH antiserum α-MSH release restored to the low control levels The inhibitory effect of MCH on tilapia melanotropes is in line with observations in trout and eel, that immunoabsorbance of endogenously released MCH enhanced the ratio of released over stored amounts of α MSH [Barber *et al*, 1987]

These findings indicate that under physiological conditions the effect of MCH on α-MSH is inhibitory

High concentrations of MCH (10 μM) and $MCH_{2\ 17}$ (10 and 35 μM) significantly increased α-MSH release The physiological importance of this stimulation of α MSH release is as yet unclear It is doubtful whether concentrations up to 10 μM MCH, which are very high compared with MCH plasma values of trout (10 to 300 pM) [Eberle *et al*, 1989, Kishida *et al*, 1989, Green & Baker, 1991, Green *et al*, 1991], occur in the teleost pituitary However, it cannot be excluded that at the MCH nerve terminals in the vicinity of the melanotropes MCH occasionally reaches micromolar concentrations We demonstrated that the stimulation of α-MSH release by MCH could not be attributed to the slight cross-reactivity of MCH with the antibody used in the α-MSH RIA The occurrence of this cross-reactivity supports the notion that the tertiary structures of α-MSH and MCH are related [Castrucci *et al*, 1989] Apparently, this structural similarity is lost when the N terminal amino acid (Asp) of MCH is removed, because $MCH_{2\ 17}$ appeared not to cross-react with the α-MSH antibody [this study] and $MCH_{2\ 17}$ exhibited almost no α-MSH-like activity on skin melanophores [Castrucci *et al*, 1989, Matsunaga *et al*, 1989] The stimulatory effect of MCH on the α-MSH release most probably is transduced by a mechanism different from that of the skin melanophores, since the effect of $MCH_{2\ 17}$ on α-MSH release from tilapia NILs was similar to that of MCH Possibly, two MCH receptor subtypes, an inhibitory and a stimulatory one, occur in the teleost pituitary However, we cannot exclude that MCH, at a high concentration, binds to receptors for other α-MSH release stimulating agents, such as TRH or CRH [Tran *et al*, 1989, Lamers *et al*, 1991], although no structural similarity with these peptides has been described

The finding that MCH interacts with the α-MSH cell not only has implications for our understanding of the roles of both cell types in background adaptation, but probably also for their functions in the response to stressors In tilapia, both cell types are responsive to acidification as a stressor [Groneveld *et al*, 1995b, Lamers *et al*, 1992], and in trout MCH secretion and biosynthesis are affected during injection and disturbance stress [Green & Baker, 1991, Baker & Bird, 1992]



Chapter 6

Identification, cellular localization and *in vitro* release of a novel teleost melanin-concentrating hormone gene-related peptide

ABSTRACT

The melanin-concentrating hormone (MCH) precursor encodes MCH and a second peptide named neuropeptide EI (NEI) in mammals, neuropeptide EV (NEV) in salmonids and MCH gene-related peptide (Mgrp) in other fish. The primary structure of the putative Mgrp of the cichlid fish tilapia (*Oreochromis mossambicus*) appears to be very different from mammalian NEI and salmonid NEV. To investigate the processing and release of tilapia Mgrp (tMgrp), in the present study an antiserum was raised against synthetic tMgrp. By immunocytochemistry, tMgrp immunoreactivity was colocated with MCH immunoreactivity in the tilapia hypothalamus and pituitary. In addition, a tMgrp enzyme-linked immunosorbent assay (ELISA) in combination with reversed phase HPLC was used to demonstrate the presence of processed tMgrp in tilapia hypothalamus and pituitary. The release of tMgrp from neuro-intermediate lobes (NILs) of tilapia pituitaries was demonstrated after *in vitro* incubation of chopped NILs. Depolarizing concentrations of potassium significantly stimulated tMgrp release. Six weeks of adaptation of tilapia to white or black backgrounds had no effect on *in vitro* tMgrp release nor on the tMgrp content of NIL and hypothalamus. Tilapia Mgrp, unlike MCH, had no effect on tilapia scale melanophores, nor did modulate the melanin concentrating effect of MCH. We conclude that tMgrp is processed from the MCH preprohormone, that it is released *in vitro*, and that the peptide has no direct role in melanin concentration of fish scale melanophores. Therefore a neuro-endocrine or neuromodulatory function is proposed for tMgrp.

With P H M Balm & S E Wendelaar Bonga
Neuroendocrinology (In press)

INTRODUCTION

Melanin-concentrating hormone (MCH) is a neuropeptide predominantly synthesized in the hypothalamus In teleosts most hypothalamic axons project to the pituitary, where the peptide is stored and released [Baker, 1991] In these fish, MCH was first discovered as a neurohypophysial hormone involved in the regulation of background adaptation More recently functions in the regulation of the stress response have been attributed to this cyclic peptide in teleosts [Baker, 1991, 1992, Groneveld *et al*, 1995b] In mammals MCH containing fibers are broadly distributed throughout the central nervous system [Bittencourt *et al*, 1992], and functions in osmoregulation, stress response and lactation have been attributed to mammalian MCH [Baker, 1991, 1994]

Sequence analysis of MCH preprohormones of teleosts such as salmonids [One *et al*, 1988, Minth *et al*, 1989, Nahon *et al*, 1991, Baker *et al*, 1995] and tilapia [Groneveld *et al*, 1993], and of mammals [Nahon *et al*, 1989, Presse *et al*, 1990, Breton *et al*, 1993a, 1993b], revealed the presence of putative neuropeptides in the prohormone in addition to MCH MCH is located at the carboxy-terminus of the prohormone and is highly conserved during evolution The peptide directly preceding MCH in the prohormone is named neuropeptide glutamic acid-isoleucine amide (NEI) in mammals [Nahon *et al*, 1989, Presse *et al*, 1990, Breton *et al*, 1993a], neuropeptide glutamic acid-valine (NEV) in salmonids [Nahon *et al*, 1991, Baker *et al*, 1995] and MCH gene-related peptide (Mgrp) in tilapia [Groneveld *et al*, 1993] In the mammalian MCH prohormone another putative peptide named neuropeptide glycine-glutamic acid (NGE) or neuropeptide proline-glutamic acid (NPE) precedes NEI NEI and NGE/NPE are highly conserved between man, rat and mouse (85 to 100 % identity), while the conservation between mammalian and salmonid NEI and NEV is considerably lower (30 % identity and 65 % similarity) However, the primary structure of the putative Mgrp of the teleost tilapia is clearly different in length and amino acid sequence if compared with the structures of the other known peptides (Fig 6 1)

Recently evidence has been provided that mammalian NEI is processed from the MCH prohormone and that the peptide is released *in vitro* [Parkes & Vale, 1992] Release of both rat NEI and MCH was stimulated by the secretagogues cAMP and cGMP, whereas only MCH release was increased by dexamethasone [Parkes & Vale, 1992] Biological actions of NEI are partly similar to and partly different from that of mammalian MCH [Baker, 1994] Both peptides may contribute to lactation, since they stimulate oxytocin release from rat pituitary glands *in vitro* Only NEI inhibits vasopressin release from rat pituitaries, indicating an involvement in osmoregulation [Parkes & Vale, 1993]

The questions arise whether tilapia Mgrp (tMgrp) is processed from the MCH prohormone, whether this Mgrp is released and whether it has a function as a neurohormone

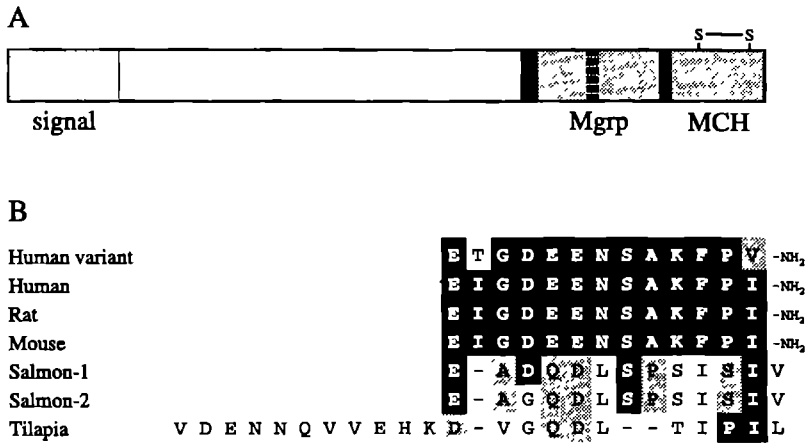


Figure 6.1 Schematic representation of MCH preprohormone, and comparison of mammalian NEI, salmon NEV and tilapia Mgrp. **A.** MCH preprohormone of tilapia. MCH, Mgrp and the signal peptide are noted. Black bars indicate two or three basic residues; the interrupted bar indicates the site where in mammals and salmonids NEI and NEV are processed, S-S, disulfide bond between two cysteins. **B.** Comparison of human NEV variant [13], human [11], rat [10], mouse NEI [12], salmon-1 and -2 NEV [6], and tilapia Mgrp [3]. Amino acids identical to mammalian NEI are black boxed, conservative amino acid substitutions are grey boxed.

in fish. Therefore, in the present study an antiserum was raised against synthetic tMgrp, and subsequently tMgrp immunoreactivity was localized in the brain and compared with the location of MCH immunoreactivity. Furthermore, the presence of processed peptide in tilapia hypothalamus and pituitary, and the *in vitro* release of tMgrp from pituitary neuro-intermediate lobes (NILs) were investigated by an immunosorbent assay and HPLC analysis. To study whether Mgrp, like MCH, has a function in background adaptation, the Mgrp release from NILs was compared in white- and black-adapted tilapia, and the *in vitro* effect of Mgrp on scale melanophores was determined.

MATERIALS & METHODS

Fish

Tilapia of both sexes were bred in the laboratory and fed twice daily commercial dried fish food (Tetramin) The fish were held at 26 °C in continuously aerated and filtered freshwater under a 12 h light, 12 h dark cycle For all experiments sexually mature males or females were used Body weight ranged from 70 to 200 g After the experiments the animals were sacrificed by spinal transection The brain and pituitary were removed

Background adaptation

Male tilapia were adapted to white or black backgrounds as described previously [Groneveld *et al*, 1995a] After six weeks the fish were netted and sacrificed

Synthetic tilapia Mgrp

Tilapia Mgrp was synthesized at the Department of Organic Chemistry of our University by solid phase peptide synthesis [Merrifield, 1963] using the Fmoc strategy for cleavage [Chang & Meienhofer, 1978] Synthetic tMgrp was coupled to bovine serum albumin (tMgrp-BSA) and to casein (tMgrp-Cas) by carbodiimide [Stewart & Young, 1984] The conjugate was diluted in 0 1 M phosphate buffer, pH 7 0, and stored in aliquots at -20 °C

Tilapia Mgrp antiserum

Antiserum to tMgrp was raised by Eurogentec (Seraing, Belgium) The antigenic conjugate, tMgrp-BSA (0 1 mg/0 2 ml), was emulsified in an equal volume of Freund's complete adjuvant and used for immunization of a rabbit by intradermic multisite injection The rabbit was boosted at day 14, 28, 56, and 84, and bled 10 days after each injection from boost 2 Sera were collected and stored at -20 °C

Immunocytochemistry

Brains obtained from tilapia kept at a neutral background were fixed overnight in Bouin's fluid, dehydrated, and embedded in paraffin Five µm thick sections were mounted on poly-L-lysine coated microscope slides Alternating sections were used for either MCH or tMgrp immunocytochemistry MCH immunocytochemistry was performed as described before [Groneveld *et al*, 1995a] Immunocytochemistry with tMgrp antiserum was performed following the same procedure with an antiserum dilution of 1 1000 For specificity control the MCH and tMgrp antisera were preadsorbed with 1 µM synthetic MCH (Peninsula) or 1 µM tMgrp prior to incubation on the slides Sections were examined with bright field microscopy

Nomenclature of brain areas was based on the work of Batten and coworkers [Batten *et al*, 1990]

***In vitro* static incubation and tissue extraction**

Static incubation of tilapia NIL fragments was performed following a procedure modified from the one described for goldfish [Yu *et al*, 1991] NIL fragments were obtained by cutting the freshly prepared NILs four times in different directions NIL fragments were washed in 1 ml of incubation medium (IM, 142 mM NaCl, 2mM KCl, 2 mM $CaCl_2$, 15 mM HEPES (pH 7 38), 2 5 mg/ml glucose, carbogen aerated) and pre-incubated in 400 µl IM in a 24 wells plate for 1 h at 28 °C All incubations were performed in a shaking waterbath After the pre-incubation the medium was replaced with 400 µl fresh IM and the tissue was incubated for 90 min at 28 °C Then the medium was refreshed with IM containing 60 mM KCl or normal IM (control), followed by 90 min incubation at 28 °C At the end of the incubation, media were transferred to 1 5 ml eppendorf tubes and stored at -20 °C until ELISA for immunoreactive tMgrp (ir-tMgrp) Stability of tMgrp was confirmed by incubating 8 or 2 µg of synthetic tMgrp in 400 µl IM under the same conditions as the tissue No significant differences were found between samples before and after incubation The tissue recovered from the incubation well or freshly dissected tissue was homogenized in 300 µl 0 01 N HCl and the supernatant obtained after centrifugation was stored in aliquots at -20 °C

Reversed phase-HPLC analysis

To determine the processing of tMgrp, extracts of tilapia NIL and hypothalamus were submitted to HPLC analysis on a Spherisorb 10 ODS column (Bisschof) The primary solvent was buffer B (0 5 M formic acid, 0 14 M pyridine, pH 3 0) and elution was accomplished with a gradient of 1 propanol at a flow rate of 2 ml/min [Martens *et al*, 1981] Fractions of 1 to 4 ml were collected The fractions were dried in a speed-vac concentrator (Savant), diluted in dilution buffer for tMgrp ELISA The fractions measured were derived from 0 7 NIL-equivalent and 2 5 hypothalami Synthetic tMgrp was used as a standard

ELISA for tMgrp

A competitive ELISA was developed for tMgrp quantitation This technique is based on competition for the tMgrp antibodies between free tMgrp in standard or samples and tMgrp immobilized on microtiter plates Wells of microtiter plates (Nunc-Immuno Plate Maxisorp™, Nunc) were coated with 100 ng tMgrp-Cas in 200 µl of coating buffer (0 05 M sodium bicarbonate buffer, pH 9 6), except for the blanks which received only coating buffer Coating lasted for 1 h at 37 °C, followed by overnight storage at 4 °C Between incubation steps, coated plates were 3 times rinsed with 300 µl washing buffer (0 01 M sodium phosphate

buffered saline, PBS, pH 4 4, with 0 05 % Tween-20, Bio-Rad) The tMgrp antiserum was pre-incubated in dilution buffer (washing buffer containing 2 % porc serum) with 4 mg/ml BSA fot 2 h The antigen was diluted in dilution buffer, tissue extracts were lyophilized and diluted in dilution buffer To incubation medium samples 2 % porc serum was added For competition equal volumes of antigen and diluted antiserum (1 5000) were incubated in 1 5 ml eppendorf tubes for 16 h at 20 °C Tubes containing diluted antiserum only were incubated under the same conditions After washing the wells of coated plates were blocked with 200 µl blocking buffer (PBS containing 6 % porc serum) for 2 h at 37 °C The plates were washed and filled with 100 µl per well of the antigen/antiserum mixture (each sample in duplicate), except for the blanks and B_0s, which received 100 µl of diluted antiserum Plates were incubated for 1 h at 37 °C The wells were washed and incubated with 100 µl of goat anti-rabbit peroxidase conjugate (Nordic) in dilution buffer (1 5000) for 1 h at 37 °C For quantification of the immunoconjugate bound to the wells, an enzymatic reaction was performed with o-phenylene-diamine (OPD, Sigma) as a substrate After washing, 190 µl of substrate (0 05 % OPD in citrate/phosphate buffer, 0 2 M Na_2HPO_4, 0 1 M citric acid, pH 5 0, with 0 025 % hydrogen peroxide) was added per well The reaction was allowed to proceed for 5 - 10 min and was stopped by adding 50 µl of 4 M H_2SO_4 Absorbance was measured at dual wavelengths (490 and 655 nm) in a microplate reader (Titertek) In the ELISA the detectable range was 8 pg to 8 ng per tube Interassay variation was 14 3 % ± 7 1 % (n = 4) No cross-reactivity (0 02 %) was found with MCH (Bachem), α-melanocyte stimulating hormone (α-MSH, mono-acetylated α-MSH from Bachem), casein or cortisol (both from Sigma) Cross-reactivity with ovine corticotropin-releasing hormone was 0 1 % To test parallelism of dilution curves the computer program PHARM/PCS-version 4 1 [Tallarida & Murray, 1986] was used

Tilapia scale bioassay

Melanin-concentrating activity was estimated by an *in vitro* scale bioassay slightly modified from one described before by Kawazoe and coworkers [1987] Scales were taken from male tilapia and incubated in incubation medium with 0 3 mg/ml BSA (IMB) for 5 min at 20 °C followed by another 5 min incubation at 20 °C with IMB containing 100 nM α MSH, which is a physiological antagonist of MCH, known to act through its own specific α-MSH receptor Four or five α MSH pretreated scales, in which the melanin granules were fully dispersed, were incubated in test solutions containing α-MSH, Mgrp and/or MCH dissolved in IMB After 30 min the scales were examined under a dissection microscope, and the number of melanophores with fully aggregated granules, and the total number of melanophores were determined for each scale For each scale melanin-aggregating activity was expressed as the percentage of melanophores with fully aggregated granules after 30 min

Data analysis

Data are presented as the mean + or ± SEM For statistical analysis the paired or unpaired Student's *t* test or one-way ANOVA were used where appropriate $P < 0.05$ was accepted as indicating significant differences

RESULTS

Cellular colocalization of Mgrp- and MCH-immunoreactivity

Immunocytochemical analysis revealed tMgrp immunoreactive perikarya in the hypothalamus and pituitary (Fig 6 2) Three groups of cells could be distinguished Many large cell bodies, 12-20 μm in diameter, were located ventrally in the hypothalamus in the nucleus lateralis tuberis (NLT, Fig 6 2B) Scattered small cell bodies (4-6 μm in diameter) were found near the lateral ventricle in the nucleus recessus lateralis (NRL, Fig 6 2D) Few Mgrp-immunoreactive cell bodies (about 12 μm in diameter) were observed in the neurohypophysis (Fig 6 2F) The majority of staining in the neurohypophysis was found in axonal nerves derived from Mgrp-containing hypothalamic neurons (Fig 6 2F) Most of the tMgrp positive fibers penetrated the intermediate lobe of the pituitary, while some penetrated the anterior lobe Mgrp-immunoreactive fibers were also observed in the brain, predominantly in the hypothalamus and in the ventral telencephalon When tMgrp-antiserum preadsorbed with synthetic tMgrp was used, the staining in brain and pituitary was greatly reduced, whereas preadsorption of tMgrp with MCH-antiserum, and of MCH with tMgrp-antiserum had no effect on staining with the respective antisera (not shown) Immunoreactivity of tMgrp in hypothalamic NLT and neurohypohpysial cells was colocated with MCH immunoreactivity (Fig 6 2A,B,E,F) Because of the small size of the Mgrp- and MCH-immunoreactive perikarya in the NRL, it was not possible to stain the same cells in alternate sections (Fig 6 2C,D) The tMgrp and MCH staining of neurohypophysial axonal nerves was in the same region (Fig 6 2E,F)

Identification of tilapia Mgrp

The identity of tMgrp immunoreactivity was confirmed by two approaches Firstly, the dilution curves of tMgrp immunoreactivity of tilapia NIL extracts and media paralleled that of synthetic tMgrp in the tMgrp ELISA (Fig 6 3) In both cases the slope of the log transformed curves was not significantly different from the tMgrp standard curve Secondly, qualitative analyses of NIL and hypothalamus extracts by reversed phase HPLC revealed a single peak immunoreactive for tMgrp as measured by ELISA The retention time (20 3 min) of this peak

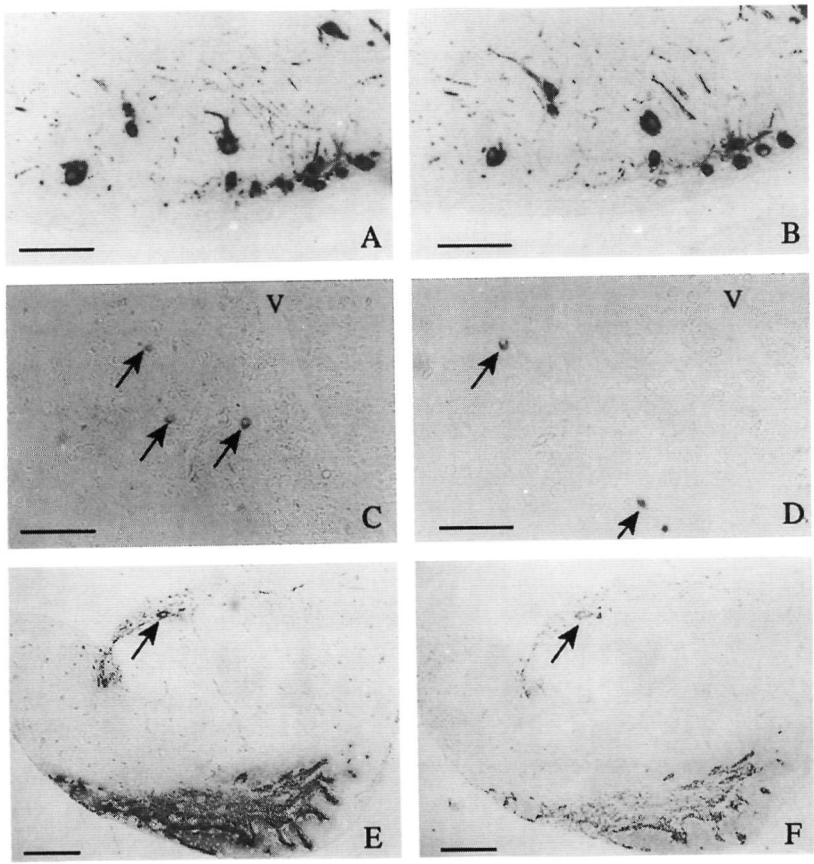


Figure 6.2 Immunocytochemical colocalization of Mgrp and MCH in tilapia hypothalamus and pituitary. **A** and **B**, **C** and **D**, and **E** and **F** are alternating sections. **A**, **C** and **E** are stained with MCH-antiserum, and **B**, **D** and **F** with Mgrp-antiserum. **A** and **B**, NLT; magnocellular neurons contain both MCH and Mgrp, scale bars are 50 μm. **C** and **D**, Small cells immunoreactive for MCH and Mgrp (arrows) occur near the lateral ventricle (V) in the NRL, scale bars are 50 μm. **E** and **F**, pituitary. Mgrp and MCH immunostaining is visible in the neurohypophysis, predominantly in axons and also in a cell body (arrow); scale bars are 100 μm.

was identical to that of synthetic tMgrp (Fig. 6.4). No signal was found in the tMgrp ELISA when trout or carp NIL extracts were used.

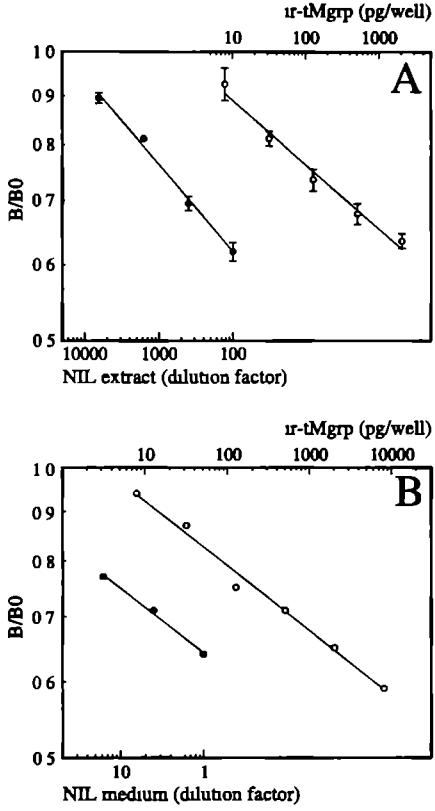


Figure 6.3 Standard and displacement curves in Mgrp ELISA **A.** Tilapia Mgrp standard curve in dilution buffer (○, n = 4) and dilution of NIL extract (•, n = 6) **B.** Tilapia Mgrp standard curve in incubation medium (○) and dilution of NIL incubation medium (•) Each point represents the average of duplicate determinations

Release and tissue content of tMgrp

The tMgrp ELISA was used to measure the tMgrp content of medium in which chopped NILs had been incubated *in vitro* Tilapia Mgrp was detectable in the medium of all NIL samples, indicating that *in vitro* tMgrp is released Significant increases in the release of tMgrp were measured after incubation of NILs in the presence of 60 mM K^+, whereas in controls the tMgrp release was not significantly changed (Fig 6 5A) After incubation, a considerable amount of tMgrp was still present in the NIL tissue (169 3 ± 34 2 ng/NIL, n = 5) No significant differences were found in the release from NILs (Fig 6 5B), or in tissue contents of NIL and hypothalamus (Table 6 1) between white- or black adapted fish The NIL/hypothalamus ratios of tMgrp tissue content were not significantly different between white- (18 2 ± 5 1) and black-adapted fish (13 7 ± 3 8)

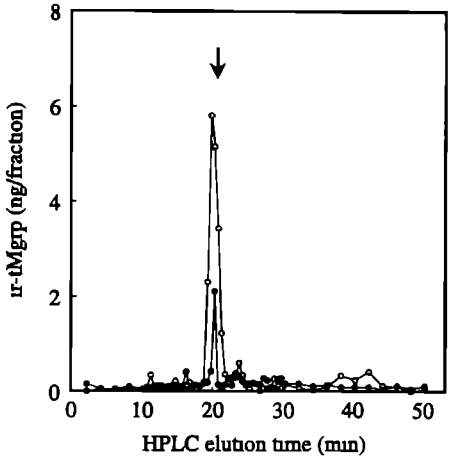


Figure 6 4 Reversed phase HPLC analysis of tMgrp immunoreactivity in NIL (○) and hypothalamus (•) extracts The retention time of synthetic tMgrp is shown by an arrow

Effect of tMgrp on tilapia scales

Melanin-concentrating activities of tMgrp, MCH and combinations of both peptides are compared in Fig 6 6 Concentrations of 10 nM to 10 μM synthetic tMgrp had no melanin concentrating effect in contrast to 1 to 100 nM MCH When combinations of tMgrp and MCH were added, the effect was similar to the effect of only MCH addition These findings indicate that tMgrp has no melanin-concentrating activity, and does not influence the effect of MCH

DISCUSSION

The present results provide evidence that tilapia Mgrp, a cleavage product of the MCH preprohormone, is present in the NIL and hypothalamus of this fish, and that tMgrp is released *in vitro* Furthermore, tMgrp appeared to play no direct role in melanin concentration in fish scale melanophores

The first indication that tMgrp is synthesized in tilapia brain and pituitary was the colocation of tMgrp immunoreactivity with MCH immunoreactivity Tilapia Mgrp immunoreactivity was detected in all regions previously reported to contain MCH and ppMCH mRNA [Groneveld *et al* , 1995a] The location of Mgrp in the tilapia hypothalamus greatly corresponds with the recently reported cellular localization of trout MCH and ppMCH mRNA

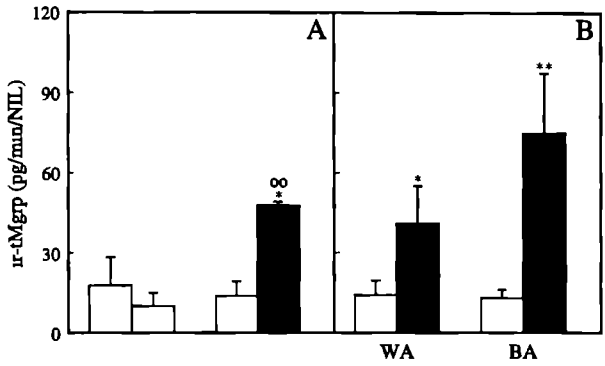


Figure 6.5 *In vitro* release of tMgrp in response to background colour and depolarizing concentrations of potassium from NIL fragments Empty bars represent incubation medium with standard K^+ (2 mM), filled bars represent medium with high K^+ (60 mM) Linked bars represent subsequent incubations of 90 min each **A.** Effect of high K+ on tMgrp release from NILs of tilapia kept on a neutral background Three NILs were incubated for two subsequent periods in standard K^+ (left), another three were exposed to high K^+ during the second incubation period (right) **B.** Effect of background colour on Mgrp release W, NIL of white-adapted tilapia (n = 5), B, NIL of black-adapted tilapia (n = 5) * $P < 0.05$, ** $P < 0.01$ compared with standard K^+ incubation of same NIL by paired Student's *t* test after log transformation of data where appropriate oo $P < 0.01$ compared with the second incubation with standard K^+ by unpaired Student's *t* test

Table 6.1 Mgrp content of NIL and hypothalamus of tilapia adapted white and black backgrounds for 6 weeks, n = 5

		Mgrp (ng/gland)	
	BW (g)	**NIL**	**hypothalamus**
White	74 8 ± 7 9	237 0 ± 84 1	14 2 ± 3 4
Black	99 4 ± 5 5	204 2 ± 40 0	17 4 ± 3 2

[Baker *et al* , 1995] In both fish species magnocellular neurons in the NLT are stained, while near the lateral ventricle a group of smaller cells occurs However, the tilapia NRL neurons appeared to be located at the caudal site of the lateral ventricle, whereas the trout NRL neurons are situated near the dorsal surface Secondly, Mgrp immunoreactivity in tilapia NIL and hypothalamus was, by criteria of HPLC elution profiles and ELISA studies,

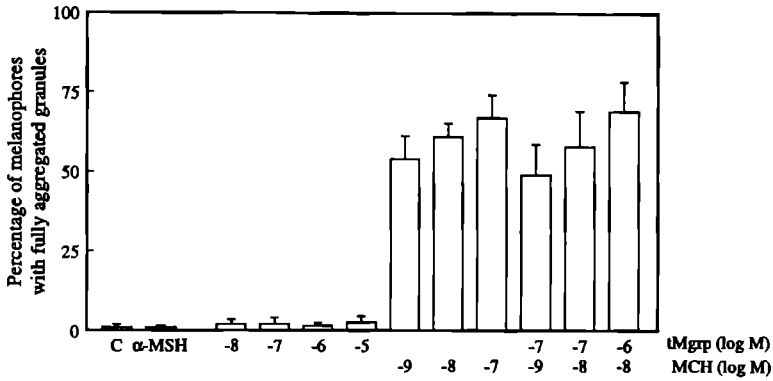


Figure 6.6 Effect of synthetic tMgrp on melanin aggregation of tilapia scale melanophores. Mgrp and MCH concentrations are shown in the figure. α-MSH concentration was 100 nM. IMB, incubation medium containing BSA. Number of scales was 4 or 5. All samples containing MCH were significantly different from samples without MCH ($P < 0.001$ by ANOVA). The experiment was repeated with similar results.

indistinguishable from synthetic tMgrp; the sequence of the synthetic peptide was based on the sequence predicted from the tilapia MCH preprohormone [Gröneveld *et al.*, 1993]. This finding indicates that a tMgrp peptide of 22 amino acids, which differs considerably in length and amino acid sequence from other MCH precursor associated peptides (Fig. 6.1), is actually processed from the tilapia MCH preprohormone. This is the first time that processing of the MCH preprohormone to a teleost Mgrp has been demonstrated. For trout it has been demonstrated by measuring MCH immunoreactivity, that the processing of the MCH preprohormone occurs via intermediates, one most probably being MCH coupled to a putative Mgrp [Bird *et al.*, 1991]. Since monobasic residues sometimes serve as cleavage sites [Schwartz, 1986], the 22-amino acids tMgrp might be further processed into two smaller forms if the lysine residue at position 11 is used as a processing site. However, it is doubtful whether this additional processing actually takes place since, according to the rules for monobasic cleavages, Lys_{11} is in an unfavourable position for cleavage [Devi, 1991]. Moreover, our antiserum probably would have recognized at least one of the two peptides resulting from such a processing. In that case the HPLC profile would have displayed more than one peak. It is not clear yet, whether the tMgrp antiserum recognizes the MCH preprohormone and/or intermediates of the processing. The HPLC profiles of NIL and hypothalamus tissue extracts contained only one clear peak, indicating that the vast majority of ir-tMgrp in these tissues is tMgrp. However, it cannot be excluded that in studies on *de novo* tMgrp synthesis, the

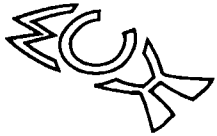
prohormone or intermediates will be recognized by the antiserum The tMgrp antiserum appeared to be specific for tilapia Mgrp, since no ir-Mgrp was detected in the tMgrp ELISA in trout or carp NIL extracts

The tMgrp contents of NIL and hypothalamus of tilapia were in the same range as values found for MCH in trout [Barber *et al*, 1987, Green *et al*, 1991] and 5 to 10 times higher than in carp [Bird & Baker, 1989] and eel [Barber *et al*, 1987] Also the ratio between tMgrp content of NIL and hypothalamus was comparable with values found for MCH in teleosts [Barber *et al*, 1987, Bird & Baker, 1989] No differences in Mgrp tissue content were found in tilapia adapted for 6 weeks to white or black backgrounds Minor differences in MCH tissue content of teleosts adapted for several weeks to white and black backgrounds have been reported before for trout and eel [Barber *et al*, 1987] A lack of difference in tissue content in itself does not mean that there are no differences in tMgrp synthesis and release between white and black-adapted tilapia, since tissue content is the overall result of synthesis, release and breakdown Therefore, Mgrp biosynthesis was indirectly determined by measuring ppMCH mRNA levels in the experimental animals, which yielded a 3 times increase in white-adapted tilapia (data not shown) as has been reported before for two to four weeks of background adaptation of this species [Groneveld *et al*, 1995a, 1995b]

To study the release of tMgrp, an *in vitro* incubation system was developed Evidence was provided that the presence of tMgrp in the incubation medium of chopped NILs was the result of regulated tMgrp release, since Mgrp secretion was enhanced in response to depolarizing concentrations of potassium This stimulatory effect of potassium and regulatory effects of other secretagogues have been reported before for the release of gonadotropin-releasing hormone (GnRH) from goldfish pituitaries in a similar experimental set-up [Yu *et al*, 1991, Rosenblum *et al*, 1994] The authors demonstrated differences in *in vitro* GnRH release between juvenile and adult fish, which appeared to be related to the GnRH content of the tissues of both group of fish [Rosenblum *et al*, 1994] In tilapia we found no differences in basal or in potassium stimulated tMgrp release between white- and black-adapted fish Thus, in analogy with the GnRH results, the *in vitro* release of tMgrp may be related to the tMgrp content of the NIL, which appeared to be similar in white- and black-adapted tilapia Probably, the regulation of tMgrp release from pituitary nerve terminals is under continuous control of hypothalamic Mgrp-perikarya or presynaptic neurons In the *in vitro* incubation system used, the tMgrp fibers in the NIL fragments are separated from their perikarya, and may explain why there is no difference in release between the tissues from white- and black-adapted fish

In contrast to MCH, the amount of tMgrp released into the circulation may be of minor importance during background adaptation, since the peptide appeared not to influence pigment dispersion of tilapia scale melanophores The effect of MCH on these melanophores was

comparable with previously reported data [Kawazoe *et al.*, 1987] Tilapia Mgrp did not influence the melanin-concentrating effect of MCH either. This indicates that tilapia Mgrp has no function similar to MCH in background adaptation, which is in line with findings in mammals, where NEI and MCH had different effects on vasopressin secretion. However, NEI and MCH appeared to have a similar role in mammalian lactation by inhibiting oxytocin release *in vitro* [Parkes & Vale, 1993]. A function for tMgrp can be sought in the control of the response to stressors such as disturbance, handling, or acid water, since these challenges are known to influence MCH biosynthesis [Baker & Bird, 1992] and/or ppMCH mRNA expression [Gröneveld *et al.*, 1995b]. Possibly, tMgrp has a neuro-endocrine or neuromodulatory role in the response to these stressors.



Chapter 7

Mgrp stimulates ACTH, but not α-MSH, release from the tilapia pituitary

ABSTRACT

Tilapia (*Oreochromis mossambicus*, teleostei) melanin-concentrating hormone gene-related peptide (tMgrp) was tested for tropic actions on adenocorticotropin hormone (ACTH) and α-melanocyte stimulating hormone (α-MSH) producing cells in the tilapia pituitary gland *in vitro* Up to 100 μM synthetic tilapia Mgrp (tMgrp) had no effect on α-MSH release from tilapia neuro-intermediate lobes in a superfusion set up However, at concentrations above 1 μM, tMgrp concentration-dependently stimulated ACTH release from tilapia anterior lobes This is the first indication that Mgrp modulates the ACTH release from teleost corticotropes, and this might imply that the peptide is involved in regulation of the pituitary-interrenal axis of fish

INTRODUCTION

Melanin concentrating hormone (MCH) was first discovered in teleostean fishes as a neurohypophysial hormone involved in the regulation of background adaptation More recently a function in the regulation of the stress response has been attributed to this peptide in teleosts [Baker 1991, Baker & Bird 1992, Groneveld 1995b] Sequence analysis of MCH preprohormones of salmonids [Minth *et al*, 1989, Ono *et al*, 1988, Baker *et al*, 1995], tilapia [Groneveld *et al*, 1993] and mammals [Nahon *et al*, 1989, Presse *et al*, 1990, Breton *et al*, 1993] indicated that processing of the prohormone may yield more functional peptides besides MCH Indeed, the actual processing of mammalian neuropeptide glutamic acid isoleucine amide (NEI) [Parkes & Vale 1992] and of tilapia MCH gene-related peptide (tMgrp) [Groneveld *et al*, 1995d] from the MCH preprohormone has been demonstrated Both peptides directly precede MCH in the prohormone Tilapia Mgrp is very different from NEI in length and amino acid sequence

[Groneveld *et al*, 1993, 1995d], in contrast to MCH itself, which is highly conserved during evolution

MCH and Mgrp are colocated in the hypothalamus and pituitary of tilapia [Groneveld *et al*, 1995d] Most MCH and tMgrp containing neurons are located in the nucleus lateralis tuberus, and they project mainly to the neurohypophysis The majority of the MCH/Mgrp containing neurohypophysial fibers penetrate in the neuro intermediate lobe (NIL), while some project to the anterior lobe (AL) In teleosts, a modulatory action of MCH on pituitary melanotropes and corticotropes has been described At physiological concentrations MCH inhibits α-MSH release from the pituitary of trout [Baker *et al*, 1986, Barber *et al*, 1987] and tilapia [Balm *et al*, 1993, Groneveld *et al*, 1995c], whereas at high concentrations MCH stimulates α-MSH release [Groneveld *et al*, 1995c] For trout an inhibitory action of MCH on CRH stimulated ACTH release has been reported [Baker *et al*, 1985, Baker *et al*, 1986], which is assumed to result from an inhibitory action of MCH on the release of CRH [Baker 1994]

In mammals, the actions attributed to NEI are partly similar to, and partly different from that of MCH [Baker 1994] both peptides may facilitate lactation by stimulation of oxytocin release from rat pituitary glands, whereas NEI, but not MCH, inhibits vasopressin release from rat pituitaries *in vitro*, indicating an involvement in osmoregulation [Parkes and Vale, 1993] The function of tMgrp is still unclear In a previous study it was shown that tMgrp has no effect on pigment dispersion of tilapia scale melanophores *in vitro*, and that it does not interfere with the pigment concentrating action of MCH [Groneveld *et al*, 1995d] The question arises whether tMgrp, like MCH, exhibits effects at the pituitary level Therefore, in the present study effects of synthetic tMgrp on α-MSH and ACTH release from the tilapia pituitary gland were investigated using an *in vitro* superfusion set up

MATERIALS & METHODS

Animals

Male tilapia (*Oreochromis mossambicus*), ranging from 15 to 25 g, were bred in the aquarium facility of the Dept of Animal Physiology of the University of Nijmegen They were kept in fresh water at 28°C and were fed a commercial dried fish food (Tetramin) The fish were kept in glass aquaria, illuminated by overhead TL tubes, with a day-night rhythm of 12 h light and 12 h darkness Immediately after removal from the tank, the

animals were sacrificed by spinal transection and pituitary glands were dissected from the brain

Superfusion of pituitary tissue

Freshly dissected NILs or ALs were superfused as described before [Groneveld *et al*, 1995c] Pulses (30 min) were given with 1 nM to 100 μM synthetic tMgrp [Groneveld *et al*, 1995d] Controls received incubation medium (142 mM NaCl, 2mM KCl, 2 mM $CaCl_2$, 15 mM HEPES (pH 7 38), 0 3 mg/ml bovine serum albumin (Sigma), 2 5 mg/ml glucose) without tMgrp The collected superfusion fractions were immediately frozen and stored at -20°C until α-MSH or ACTH radioimmunoassay

Radioimmunoassays

The α-MSH radioimmunoassay (RIA) with L9 α-MSH antiserum has been described before [Balm *et al*, 1995] The RIA for ACTH with antiserum against $ACTH_{1\ 24}$ also has been described previously [Balm *et al*, 1994] The α MSH and ACTH antisera did not crossreact with tMgrp

Processing of data and statistics

For the concentration response relationship of tMgrp-induced effects on ACTH and α-MSH release, values for maximum stimulation of ACTH or α-MSH release during the tMgrp pulse were calculated by subtracting the basal release value from the maximum pulse values for each incubation Basal release was defined as the average hormone release in 20 to 30 min prior to addition of secretagogue The presented values for maximum stimulation were corrected for the increasing basal release [Balm *et al*, 1994] by subtraction of the maximum increase of controls which were superfused simultaneously The difference between maximum stimulation in the tMgrp pulsed lobes and in controls during the same period was statistically analyzed Statistical analyses were performed using the unpaired Student's *t* test Significance was accepted at $P < 0\ 05$

RESULTS

Effects of tMgrp on ACTH and α-MSH release

The stimulatory effect of tMgrp on the ACTH release from tilapia ALs during *in vitro* superfusion lasted for the duration of the pulse, after the pulse ACTH release did return to control levels (Fig 7 1) At a concentration of 1 μM tMgrp tended to stimulate ACTH

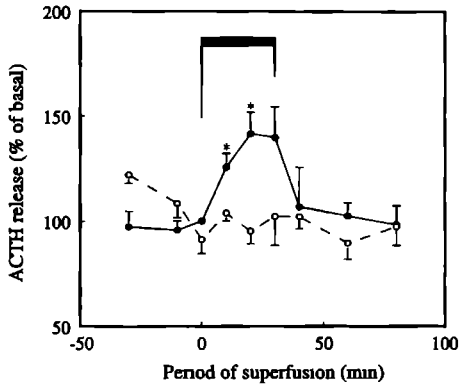


Figure 7.1 Effect of 10 μM synthetic tMgrp on ACTH release from tilapia ALs during *in vitro* superfusion expressed as percentage of basal release (= 100%) -●-, tMgrp, n = 8, basal release 3 06 ± 0 57 pg/min/AL, -O-, control, n = 4, basal release 4 15 ± 0 46 pg/min/AL The time of addition of secretagogue after 400 min of superfusion is designated t_0 * $P <$ 0 05 compared with the same fraction of control superfusion

release, although at this concentration the stimulation was not statistically significant ($P >$ 0 1) At 10 and 100 μM tMgrp caused a significant stimulation of ACTH release, which was most pronounced at 100 μM (Fig 7 2A) Concentrations lower than 1 μM had no effect Tilapia Mgrp, at concentrations from 1 nM to 100 μM, had no effect on α-MSH release of tilapia NILs (Fig 7 2B) At all concentrations tested no statistically significant differences in α-MSH release occurred between pulsed and control lobes

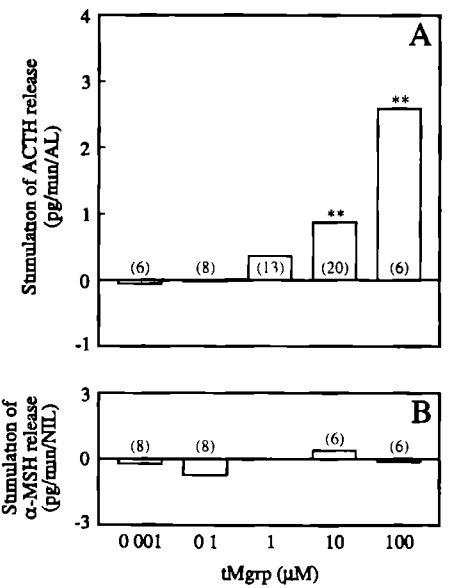


Figure 7.2 Effects of synthetic tMgrp on ACTH release from ALs and α-MSH release from NILs taken from the same fish, during *in vitro* superfusion Data are expressed as maximum stimulation during tMgrp pulse vs basal release minus maximum stimulation in control superfusion Numbers of incubations are given in parentheses for each concentration tested **A.** Concentration response relationship of tMgrp induced effects on ACTH release Basal release was 2 05 ± 0 15 pg/min/AL (n = 81) **, $P <$ 0 01 compared with control superfusion **B.** Concentration response relationship of tMgrp induces effects on α-MSH release Basal release was 6 67 ± 0 64 pg/min/NIL (n = 43)

DISCUSSION

We here demonstrate that relatively high concentrations of tMgrp stimulate ACTH release from tilapia corticotropes *in vitro*, indicating that tMgrp might modulate the pituitary-interrenal axis by a paracrine or synaptic action The peptide appeared to have no effect on α-MSH release from tilapia melanotropes

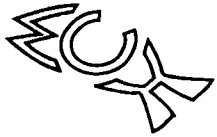
The stimulatory effect of synthetic tMgrp on ACTH release is the first demonstration that a peptide derived from the MCH preprohormone, other than MCH itself, affects ACTH release At least two lines of evidence can be advanced that native tMgrp will show the same bioactivity as synthetic tMgrp Firstly, because the amino acid sequence of synthetic tMgrp was based on the sequence deduced from native preproMCH mRNA, and secondly since the HPLC retention times of both preparations were identical [Groneveld *et al*, 1995d] The apparent influence of tMgrp on tilapia corticotropes implies the involvement of a new component of the MCH-system in stress response By preproMCH mRNA expression studies [Groneveld *et al*, 1995b], studies on MCH newly synthesis [Baker & Bird 1992] and plasma MCH measurements [Green *et al*, 1991, Green & Baker, 1991] it was demonstrated that the MCH system is responsive to stress, usually resulting in an enhancement of ppMCH mRNA expression and MCH levels However knowledge on the sites of action of MCH preprohormone-derived peptides on the hypothalamus-pituitary-interrenal (HPI) axis is limited In trout MCH has, besides its effect on CRH induced ACTH release [Baker *et al*, 1985, 1986], no effect on cortisol release from trout interrenal tissue [Green *et al*, 1991] Potentially, the effect of MCH on melanotropes in both trout [Baker *et al*, 1986, Barber *et al*, 1987] and tilapia [Groneveld *et al*, 1995c] may also have consequences for the activity of the HPI axis [Lamers *et al*, 1992] Functions of neuropeptide glutamic acid-valine (NEV), the Mgrp equivalent of trout [Baker *et al*, 1995], has not been subject of study yet

The apparently low sensitivity of tilapia corticotropes to tMgrp, may indicate that the modulation of ACTH release by tMgrp occurs by a paracrine or synaptic action Micromolar concentrations of tMgrp can only be expected in the vicinity of the tMgrp nerve terminals, where the peptide is released A similar phenomenon has very recently been described for the stimulating effect of MCH on tilapia melanotropes [Groneveld *et al*, 1995c]

The effects of Mgrp on ACTH release in tilapia are opposite to the inhibitory action of MCH on CRH stimulated ACTH release from trout pituitaries [Baker *et al*, 1985] The action in trout is consistent with the idea that in this species MCH forms part of a feedback loop, modulating CRH secretion and the HPI axis [Baker, 1994] In trout plasma cortisol values are lower in white-adapted animals than in black-adapted fish [Baker &

Rance, 1981, Green et al , 1991] The lower values in white-adapted trout were explained by the feedback effect of MCH on the HPI axis, MCH levels are elevated when fish are kept on a white background [Baker, 1994] However, in tilapia no differences were found in plasma cortisol levels between black- and white-adapted fish, while ppMCH mRNA levels were 3 times higher in white-adapted fish [Groneveld *et al*, 1995b] This is an indication that the tilapia MCH-system may partly function differently from that in trout Therefore the different effects of MCH precursor derived peptides on ACTH release from trout and tilapia probably relate to species differences

The lack of effect of 1 nM to 100 μM tMgrp on α-MSH release contrasts with the inhibition of α-MSH induced by MCH at low concentrations [Barber *et al*, 1987, Balm *et al*, 1993, Groneveld *et al*, 1995c], and the stimulation at high concentrations of MCH [Groneveld *et al*, 1995c] A possible explanation for this difference is the assumption that pituitary melanotropes of tilapia have receptors for MCH but not for Mgrp These findings together with the previously reported lack of effect of tMgrp on tilapia scale melanophores [Groneveld *et al*, 1995d] indicate that in tilapia Mgrp by itself does not exert a direct, nor an indirect function in the regulation of background adaptation However, it cannot be ruled out that tMgrp, which is most probably co-released with MCH [Groneveld *et al*, 1995d], influences the action of MCH on pituitary targets



Chapter 8

Summary & General discussion

SUMMARY OF THE MAIN RESULTS

The purpose of the research described in this thesis was to gain insight in the function of the melanin-concentrating hormone (MCH)-system in a teleost fish, in particular with respect to the peptidergic communication between hypothalamic MCH neurons and endocrine cells of the pituitary gland Therefore, firstly the structure and distribution of MCH precursor-derived peptides of tilapia, and the mRNA encoding these peptides were analyzed Using these tools the response of the tilapia MCH system to environmental challenges was investigated, and effects of MCH and MCH gene-related peptide (Mgrp) on pituitary melanotrope and corticotrope cells were studied The main results of the studies in this thesis can be summarized as follows

- Cloning and subsequent sequence analysis of the tilapia MCH preprohormone revealed the presence of MCH at the carboxy terminus and of a putative Mgrp just preceding MCH Comparison of the tilapia MCH preprohormone with its homologues in other species showed that the amino acid sequence of MCH is strongly conserved between vertebrates, whereas the putative tilapia Mgrp (tMgrp) is very different from others in length as well as in amino acid sequence [Chapter 2]
- PreproMCH mRNA expression studies and immunocytochemistry revealed the presence of tilapia ppMCH mRNA and of immunoreactive MCH and tMgrp in three nuclei of the hypothalamus and pituitary [Chapters 3 & 6], while no ppMCH mRNA was found in other tissues [Chapter 2] A large group of magnocellular MCH neurons is located in the NLT of the hypothalamus Scattered small perikarya are present in the NRL, and a few cells with a size comparable to that of the NLT neurons were found in the neurohypophysis By quantitative dot blot analysis differential regulation of ppMCH mRNA expression levels in response to environmental changes was demonstrated for the two hypothalamic nuclei [Chapter 4] MCH neurons of the NLT are activated by transfer of the fish from neutral to white background, and by severe stress evoked by exposure of the fish to acidified water (pH 3 5) The MCH neurons of the NRL, in which total ppMCH mRNA levels are

8 to 20 times lower than in the NLT, appeared to respond specifically to repeated disturbance [Chapter 4].

- The biosynthesis of tilapia Mgrp was demonstrated in Chapter 6. An antibody was raised against synthetic tMgrp, and a newly developed tMgrp ELISA together with reversed phase HPLC was used to demonstrate the presence of processed tMgrp in tilapia hypothalamus and pituitary. It was shown that tMgrp is released *in vitro*, and that, in contrast to MCH, tMgrp has no effect on tilapia scale melanophores.
- The study of the effects of synthetic MCH and tMgrp on pituitary melanotropes and corticotropes showed that MCH has a biphasic effect on α-MSH release [Chapter 5], whereas tMgrp stimulates the release of ACTH, but not of α-MSH [Chapter 7]. At low concentrations MCH inhibits α-MSH release, while concentrations above 1 μM stimulate α-MSH release. The inhibitory action of MCH mainly affected the release of mono-acetylated α-MSH, one of the most melanotropic circulating α-MSH isoforms.

LOCATION OF THE MCH-SYSTEM

In our studies on the distribution of tilapia ppMCH mRNA only signals were detected in two nuclei of the hypothalamus and in the pituitary (Fig. 8.1), but not in peripheral tissues such as ovary, intestine, liver, heart, skin and headkidney [Chapters 2 & 3]. The lack of ppMCH mRNA in peripheral tissues is in line with findings in salmon [Ono *et al.*, 1988; Minth *et al.*, 1989] as has been reported in Chapter 2 However, very recently and after publication of Chapter 2, the presence of mouse [Breton *et al.*, 1993] and rat [Hervieu & Nahon, 1995] ppMCH mRNA species have been demonstrated in peripheral tissues by PCR and Northern blot hybridization with cDNA or oligonucleotide probes. PreproMCH mRNA species were detected in testis, intestinal tract and heart, but the level of ppMCH mRNA in these tissues was much lower than in the rodent hypothalamus. However, Takahashi and coworkers [1995] could not detect ppMCH mRNA expression in peripheral rat tissues by using cRNA probes for Northern blot hybridization followed by RNase treatment, which is expected to yield more specific results than the above mentioned methods, but which is less sensitive than PCR. It is of significance to further clarify the identity of the additional mRNA species detected by Hervieu & Nahon [1995]. It is possible that also in animals other than rodents ppMCH mRNA (-like) species are present in peripheral tissues, but that in the species studied so far - mainly teleosts - their levels were beyond the detection limit of the Northern blot assays used. Future studies should elucidate this question. Another striking property of the rodent MCH-system is the recently reported *in vivo* occurrence of an antisense MCH RNA in hypothalamus as well

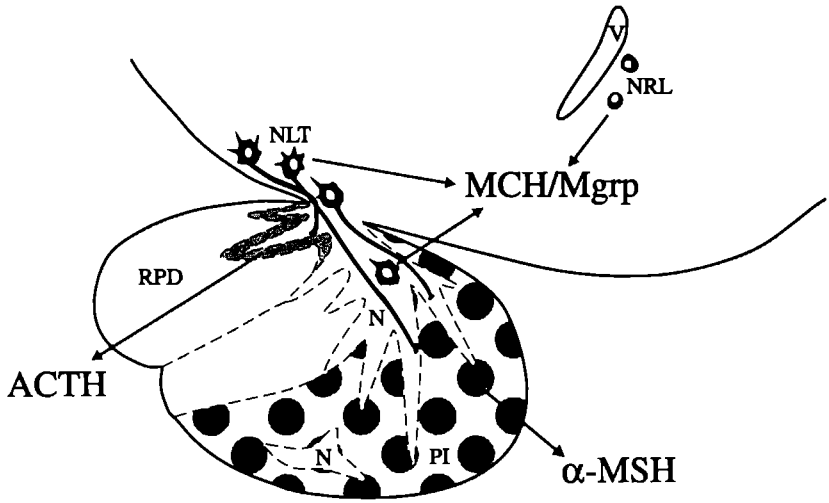


Figure 8.1 Location of the MCH-system in the tilapia hypothalamus and pituitary. MCH cells are indicated by cell-like symbols. MCH cells are present in the NLT and NRL of the hypothalamus and in the neurohypophysis. The effector sites of the MCH-system at the pituitary level, the α-MSH producing melanotropes and ACTH producing corticotropes, are grey coloured. Abbreviations: ACTH, adrenocorticotropin; α-MSH, α-melanocyte stimulating hormone, MCH, melanin-concentrating hormone; Mgrp, melanin-concentrating hormone gene-related peptide; NLT, nucleus lateralis tuberis, NRL, nucleus recessus lateralis; N neurohypophysis, PI pars intermedia; RPD, rostral pars distalis; V, lateral ventricle.

as in peripheral tissues [Hervieu & Nahon, 1995]. A role of this RNA in regulation of MCH synthesis has been suggested by these authors. If their findings can be confirmed, it will be interesting to investigate whether in tilapia and other fish MCH-systems a similar regulation takes place.

The presence of ppMCH mRNA, MCH and Mgrp in two hypothalamic regions of tilapia, the NLT and NRL, is consistent with the reported distribution of the MCH neurons in other teleosts [Naito *et al*, 1985; Batten & Baker, 1988; Baker, 1991; Baker *et al.*, 1995]. The NLT is one of the major hypophysiotropic regions of the hypothalamus. A large number of neurohypophysial factors, such as somatostatin, GABA, galanin, bombesin and to a lower extent also TRH and CRH, are produced in this region [Peter & Fryer, 1983; Batten *et al.*, 1990; Himick & Peter, 1995]. For the green molly (*Poecilia latipinna*) the exact location of the pituitary projections of fifteen neuropeptides including MCH has been established [Batten *et al.*, 1990]. The location of neurohypophysial MCH greatly corresponds with that of CRH and substance P. These three neuropeptides are mainly present close to pituitary melanotropes

and corticotropes TRH is a fourth neuropeptide present in the immediate vicinity of the melanotropes of this teleost, whereas another factor present close to corticotropes is neurotensin [Batten *et al*, 1990] These distributions in the molly indicate that both melanotropes and corticotropes are targets for several hypothalamic factors, probably leading to a complex regulation of these cells Most likely in other teleosts the distribution of these neuropeptides in the pituitary is similar, as is shown in this thesis for tilapia MCH It has been described that especially the lateral aspects of the NRL also project to the pituitary [Kah *et al*, 1993, Anglade *et al*, 1993] However, no evidence was obtained for projections of trout MCH neurons from the NRL to this gland [Baker *et al*, 1995], while in other teleosts including tilapia the projections of the MCH neurons of the NRL have not been determined yet

A FUNCTIONAL MODEL FOR THE MCH-SYSTEM OF TILAPIA

On the basis of the results of this thesis a model is postulated for the role of the tilapia MCH-system in background adaptation and stress response (Fig 8 2) In animals placed on a white background specifically MCH neurons of the NLT are activated MCH released into the circulation causes pigment concentration The process of melanin concentration in dermal melanophores on a white background in all likelihood is also effected indirectly through an inhibitory action of MCH on the α-MSH release from the pituitary As will be explained below, an attractive model for the role of the MCH system in response to severe stress is the following MCH neurons of the NLT are activated by severe stress, as for example evoked by exposure to strongly acidified water, which might result in a highly stimulated release of MCH precursor derived peptides in the pituitary Under these circumstances MCH and Mgrp concentrations may reach levels that stimulate α-MSH and ACTH release, leading to high plasma levels of these hormones and consequently cortisol In response to repeated disturbance specifically the MCH neurons of the NRL are activated, while this treatment does not activate the pituitary interrenal axis In this case the MCH-system most probably has a neuromodulatory action, as will be discussed later

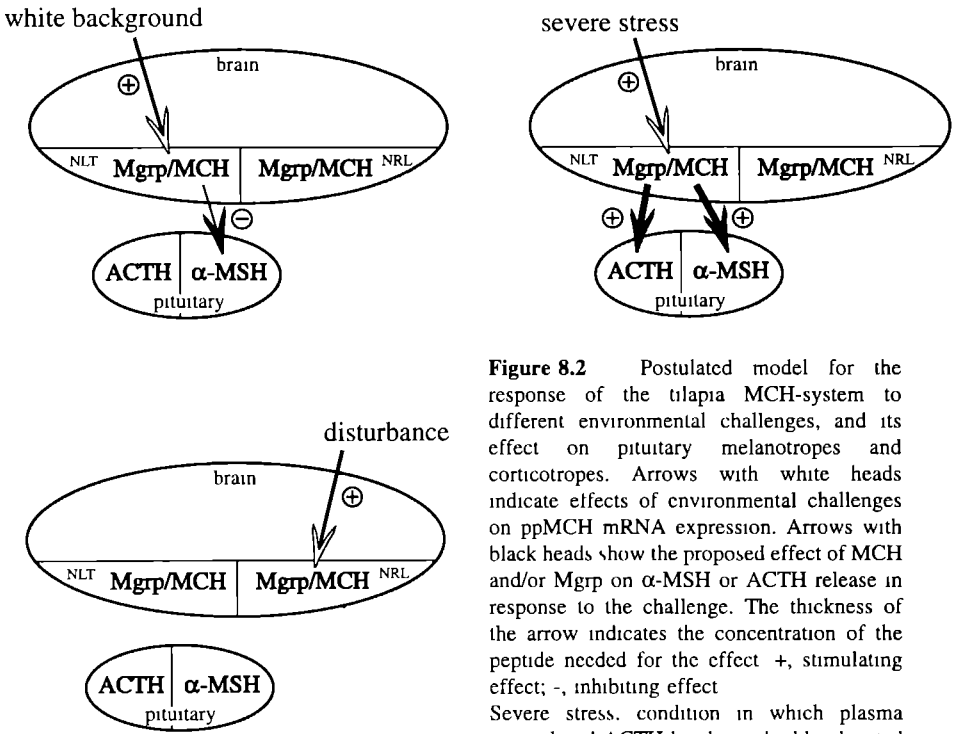


Figure 8.2 Postulated model for the response of the tilapia MCH-system to different environmental challenges, and its effect on pituitary melanotropes and corticotropes. Arrows with white heads indicate effects of environmental challenges on ppMCH mRNA expression. Arrows with black heads show the proposed effect of MCH and/or Mgrp on α-MSH or ACTH release in response to the challenge. The thickness of the arrow indicates the concentration of the peptide needed for the effect +, stimulating effect; -, inhibiting effect
Severe stress. condition in which plasma cortisol and ACTH levels are highly elevated (e.g. after exposure to strongly acidified water) Disturbance: environmental challenge not inducing a general stress response (e g. confinement).

SUBSTANTIATION OF THE FUNCTIONAL MODEL

MCH neurons of the NLT

The proposed role of the tilapia MCH-system in background adaptation is consistent with findings in other teleosts [Baker 1991; 1993]. These findings are now extended by showing that specifically MCH neurons of the NLT, but not of the NRL, are activated in response to a white background, and that MCH predominantly decreased the release of mono-acetylated α-MSH This selective decrease may be physiologically important, since the melanotropic effects of mono- and di-acetylated α-MSH are more potent than that of des-acetylated α-MSH [Rudman *et al*., 1983, Kishida *et al*., 1988].

The proposed relationship between activation of MCH/Mgrp neurons of the NLT and the stimulation of α-MSH and ACTH release during severe stress is speculative For this connection the assumption has to be made that the activation involves MCH/Mgrp release more than their biosynthesis The results of studies on the release and biosynthesis of trout MCH in response to changes in background colour and stress support this assumption It was shown that in response to a white background combined with stress the release of MCH enhanced more than in response to a white background only, and that the combined treatment resulted in the loss of MCH from both pituitary and hypothalamus [Green et al , 1991, Green & Baker, 1991] This indicates that there is more release than biosynthesis of MCH Indeed, the amount of newly synthesized MCH appeared to be only slightly increased [Baker & Bird 1992] In tilapia, only data are available on activation of ppMCH mRNA levels during severe acidification stress and white background adaptation compared to neutral background controls However, since α-MSH has an opposite effect to MCH on skin melanophores, levels of α-MSH release may be predicted to be low in response to a white background, as has been demonstrated for several teleost species [Baker, 1991, 1993] Therefore on a white background an inhibitory action of MCH on α-MSH release seems plausible

In response to mildly acidified water (pH 4 5, in the presence of aluminium) plasma α-MSH levels and *in vitro* α-MSH release were enhanced [Lamers *et al* , 1994], while after exposure to strongly acidified water (pH 3 5) also plasma ACTH values were elevated [Chapter 4] Therefore during acidification stress a stimulatory action of MCH and Mgrp on these pituitary hormones seems plausible Stimulatory actions of MCH and Mgrp only occur at relatively high, micromolar, concentrations of the peptides [Chapters 5 and 7], thus in that case high local concentrations of MCH and Mgrp are required for an effect As proposed in the previous section, it could be that these concentrations are reached during severe stress and not during adaptation to a white background, by a selective increase of MCH/Mgrp release, over and above synthesis However, this would mean that the pituitary content of MCH and Mgrp reduces, indicating that this process could only last for a limited period of time Another possibility is that during severe stress high local concentrations of MCH precursor-derived peptides occur through synaptic secretion, whereas on a white background relatively low concentrations of MCH are effective as paracrine signals A similar phenomenon has been suggested for the secretion of dopamine and its effect on α-MSH release from tilapia melanotropes at different environmental conditions [Lamers, 1994] However, this hypothesis requires plasticity of axon endings in the pituitary, a phenomenon which is currently subject of research in mammals [e g Tweedle & Hatton, 1987, Beagley & Hatton, 1992, Theodosis & Poulain, 1992], but not yet in fish In addition to MCH other hypothalamic factors affect α-MSH and/or ACTH release in response to the mentioned environmental challenges The effects of these hypothalamic factors together will determine the rate of α-MSH and ACTH

secretion. For example, a model for α-MSH release in response to exposure to acidified water proposed by Lamers [1994] describes that in acidified water, α-MSH release is stimulated by TRH, CRH and dopamine, which results in a rise of plasma α-MSH and cortisol levels. Under control conditions dopamine inhibits α-MSH release, while the tilapia melanotropes are less sensitive to CRH under these conditions.

The described functional model for the MCH-system of tilapia pays no attention to the relative importance of the MCH neurons of the NLT in the regulation of different challenges, but this will be discussed further on in this chapter.

MCH neurons of the NRL

The suggestion that in response to disturbance the activation of MCH neurons of the NRL will lead to a neuromodulatory action of the MCH-system is based on two kinds of evidence. Firstly, the applied repeated disturbance [Chapter 4] did not induce changes in plasma cortisol or α-MSH levels [Pelgrom, personal communication], which indicated that the hypothalamus-pituitary-interrenal axis is not activated during this challenge. Secondly, it has very recently been reported for trout that axons of MCH neurons of the NRL mainly project dorsally to the thalamus, whereas no evidence was obtained for projections to the pituitary [Baker, 1995]. Such projections favour a neuromodulatory role of MCH precursor-derived peptides from these NRL neurons, and this is consistent with our data.

The responsiveness of MCH neurons of the NRL to disturbance is the first indication for a regulatory role of these MCH neurons. Involvement of the posterior hypothalamus, including the NRL in feeding and aggressive behaviour has been established in earlier research involving electrical stimulation of posterior hypothalamic nuclei [Demski & Knigge, 1973; Demski 1971]. Other neuropeptides known to be present in the NRL of teleosts are somatostatin, enkephalin, bombesin and a few CRH and TRH neurons [Batten *et al.*, 1990; Kah *et al.*, 1993; Himick & Peter, 1995], and for example for bombesin a role in the central regulation of food intake has been proposed [Himick & Peter, 1995]. It could well be that the function of the MCH neurons of the NRL has been conserved during evolution. In mammals MCH neurons are located centrally and posteriorly in the hypothalamus and they innervate several brain regions, comparable to the MCH neurons in the NRL of teleosts. In the mammalian system the most innervated regions are assumed to play a role in sensorimotor integration, and are therefore believed to be involved in motivational drives associated with eating, drinking and arousal [Bittencourt *et al.*, 1992; Baker, 1994]. For the response of the mammalian MCH-system to lactation it has been described that a subpopulation of MCH neurons in the anterior hypothalamus becomes activated [Knollema *et al.*, 1992]. Possibly, the responsiveness of the mammalian MCH-system to osmotic challenges [Zamir *et al.*, 1986; Nahon *et al.*, 1993; Presse

& Nahon, 1993, Fellmann *et al*, 1993], stress [Presse *et al*, 1992, Jezova *et al*, 1992], auditory stimuli [Miller *et al*, 1993], and arousal is also regulated by subgroups of mammalian hypothalamic neurons [Baker, 1994] It could be that MCH neurons in the teleost NRL are the fish homologue of a mammalian subgroup associated with arousal, since the responsiveness of tilapia NRL neurons to disturbance might be based on the motivational aspect to escape confinement, a component of the disturbance applied [Chapter 4] Further research is needed to confirm this hypothesis However, if the link between a subpopulation of mammalian MCH neurons and the teleost NRL neurons can be established, the tilapia NRL can be used as a comparative model for further studies, because we showed that the activity of the MCH neurons in the NRL of this teleost can be investigated separately and quantitatively

COMPARATIVE RESPONSIVENESS OF THE MCH-SYSTEM TO DIFFERENT CHALLENGES

The MCH neurons of the tilapia NLT are readily activated by placing the fish on a white background, indicating that in tilapia, as in other teleosts, the primary trigger of MCH neurons in the NLT is the background colour Other challenges only stimulated the MCH neurons of the tilapia NLT at intensity levels that induced severe stress With respect to the responsiveness of the MCH-system to stress, our data are in line with observations in trout, the only other teleost species in which the role of MCH in the stress response has been studied In trout newly synthesized MCH was slightly enhanced in response to repeated exposure to stressors [Baker & Bird 1992] Thus, in both species the stimulation of MCH biosynthesis in the ventral hypothalamus was limited

Another interesting topic is the importance of MCH and α-MSH in background adaptation Our data show that changes in MCH mRNA levels are pronounced in response to changes in background colour Morphological analysis demonstrated activation of α-MSH producing melanotropes in response to a black background [Van Eys, 1981] However, in our background adaptation experiments pituitary levels of α-MSH, measured by radioimmunoassay [unpublished results] and of tMgrp, which presumably is present in quantities comparable with that of MCH [Chapter 6], did not differ between tilapia adapted to black and white backgrounds Also plasma α-MSH levels appeared to be similar in white- and black-adapted fish An explanation for the apparent discrepancy between these data may be the differences in experimental conditions Van Eys kept the fish during the experiments separately in buckets and replaced the water every other day, which was probably stressful to the fish In contrast,

we housed our fish in groups, which were not disturbed, and the water was continuously filtered Another explanation for the difference between the morphological data and our results is the possibility that in white- and black-adapted tilapia both release and turnover of the hormones are changed, while the net pituitary peptide levels and plasma α-MSH concentrations do not differ

We found that when in strongly acidified water tilapia MCH neurons of the NLT were activated, the (light) skin colour of the fish did not change Unfortunately, we were unable to measure plasma MCH values Similarly, in acid water plasma α-MSH levels are elevated [Lamers *et al* , 1994], whereas no evident darkening of the skin was observed [Lamers, 1994] On the basis of the above findings it could be postulated that MCH has an important function in background adaptation, whereas α-MSH primarily functions as a stress hormone However, it is clear from our data and the findings in trout that the MCH system is also responsive to severe stress Analysis of a precise role of MCH and Mgrp during stress response would be an interesting subject for future research Especially, comparison of the relative importance of these peptides with other hypothalamic factors involved will lead to a better understanding of the regulation of the stress response

FUTURE PROSPECTS: IDENTIFICATION OF MCH RECEPTORS

In addition to the aforementioned suggestions for further research, an important step forward in MCH research will be made by the isolation and characterization of MCH and Mgrp receptors Identification of these receptors would give more insight in the precise target sites of these MCH precursor-derived peptides in teleosts as well as in other vertebrates Localization of MCH and Mgrp receptors in the brain and pituitary gland will contribute to the elucidation of the neuromodulatory and neuro-endocrine functions of these peptides Characterization of receptors in the teleost pituitary will also elucidate whether the biphasic actions of MCH on pituitary melanotropes of tilapia and on auditory gating in rats [Miller *et al* , 1993] are caused by different receptors or by different states of the same receptor

An important step in the characterization of MCH receptors has very recently been made by the demonstration of binding a MCH radioligand to mouse melanoma cells [Drozdz *et al* , 1995], hypothalamus, hippocampus and adrenal gland [Drodz & Eberle 1995] By using such a MCH receptor binding assay combined with a molecular biological isolation strategy MCH receptor cDNA of mammals and teleosts can be cloned and identified in the near future This opens new perspectives for the research on neuro endocrine and neuromodulatory peptidergic communication of MCH neurons in teleosts

References

ANGLADE I, ZANDBERGEN T, KAH O. (1993). Origin of the pituitary innervation in the goldfish. *Cell Tissue Res* 273: 345-355

BAGNARA JT, HADLEY ME. (1973). Chromatophores and color change: the comparative physiology of animal pigmentation. Englewood Cliff, NJ: Prentice Hall.

BAKER BI, BALL JN. (1975). Evidence for a dual pituitary control of teleost melanophores. *Gen Comp Endocrinol* 25 147-152.

BAKER BI, RANCE TA. (1981). Differences in concentration of plasma cortisol in the trout and eel following adaptation to black or white backgrounds *J Endocrinol.* 89 135-140

BAKER BI, BIRD DJ, BUCKINGHAM JC. (1985). Salmonid melanin-concentrating hormone inhibits corticotropin release. *J Endocrinol.* 106: R5-R8.

BAKER BI, BIRD DJ, BUCKINGHAM JC. (1986) Effects of chronic administration of melanin concentrating hormone on corticotropin, melanotropin and pigmentation in the trout. *Gen Comp Endocrinol.* 63: 62-69.

BAKER BI. (1991) Melanin-concentrating hormone. a general vertebrate neuropeptide *Int Rev Cytol* 126 1-47

BAKER BI, BIRD DJ. (1992). The biosynthesis of melanin-concentrating hormone in trout kept under different conditions of background colour and stress, as determined by an *in vitro* method. *J Neuroendocrinol* 4: 673-679.

BAKER BI. (1993). The role of melanin-concentrating hormone in color change. *Ann NY Acad Sci.* 680 279-289

BAKER BI. (1994). Melanin-concentrating hormone updated. Functional considerations. *Trends Endocrinol Metabol.* 5. 120-126

BAKER BI, LEVY A, HALL L, LIGHTMAN S. (1995) Cloning and expression of melanin-concentrating hormone genes in the rainbow trout brain. *Neuroendocrinology* 61: 67-76.

BALM PHM. (1986) Osmoregulation in teleosts by cortisol and prolactin, adaptation to low pH environments. *Ph D Thesis.* University of Nijmegen, Nijmegen, The Netherlands.

BALM PHM, GRONEVELD D, LAMERS AE, WENDELAAR BONGA SE. (1993). Multiple actions of melanotropic peptides in the teleost *Oreochromis mossambicus* (Tilapia). *Ann NY Acad Sci.* 680 448-450.

BALM PHM, PEPELS P, HELFRICH S, HOVENS MLM, WENDELAAR BONGA SE. (1994) Adrenocorticotropic hormone (ACTH) in relation to interrenal function during stress in tilapia (*Oreochromis mossambicus*). *Gen Comp Endocrinol* 96: 347-360.

BALM PHM, HOVENS MLM, WENDELAAR BONGA SE. (1995). Endorphin and MSH in concert form the corticotropic principle released by tilapia (*Oreochromis mossambicus*; teleostei) melanotropes. *Peptides* 16. 463-469.

BARBER LD, BAKER BI, PENNY JC, EBERLE AN. (1987). Melanin concentrating hormone inhibits the release of αMSH from teleost pituitary glands *Gen Comp Endocrinol.* 65: 79-86

BATTEN TFC, BAKER BI. (1988). Melanin-concentrating hormone (MCH) immunoreactive hypophysial neurosecretory system in the teleost *Poecilia latipinna*: light and electron microscopic study. *Gen Comp Endocrinol* 70: 193-205.

BATTEN TFC, CAMBRE ML, MOONS L, VANDESANDE F. (1990). Comparative distribution of neuropeptide-immunoreactive systems in the brain of the green molly, *Poecilia latipinna. J Comp Neurol.* 302. 893-919

BEAGLEY GH, HATTON GI. (1992). Rapid morphological changes in supraoptic nucleus and posterior pituitary induced by a single hypertonic saline injection. *Brain Res Bull.* 28 613-618.

BIRD DJ, BAKER BI. (1989). An immunological study of the secretory activity of neurons producing melanin-concentrating hormone in a teleost *Neuroscience* 28: 245-251.

BIRD DJ, BAKER BI, KAWAUCHI H. (1989). Immunocytochemical demonstration of melanin-concentrating hormone and proopiomelanocortin-like products in the brain of the trout and carp *Gen Comp Endocrinol* 74: 442-450.

BIRD DJ, BAKER BI, EBERLE A, SWANN RW. (1990). The biosynthesis of melanin-concentrating hormone in a fish. *J Neuroendocrinol.* 2. 309-315.

BITTENCOURT JC, PRESSE F, ARIAS C, PETO C, VAUGHAN J, NAHON J-L, VALE W, SAWCHENKO PE. (1992). The melanin-concentrating hormone system of the rat brain: an immuno- and hybridization histochemical characterization. *J Comp Neurol.* 319· 218-245.

BRETON C, PRESSE F, HERVIEU G, NAHON J-L. (1993a). Structure and regulation of the mouse melanin-concentrating hormone mRNA and gene. *Mol Cell Neurosci.* 4. 271-284.

BRETON C, SCHORPP M, NAHON J-L. (1993b) Isolation and characterization of the human melanin-concentrating gene and a variant gene. *Mol Brain Res* 18: 297-310.

BLALOCK JE. (1989). A molecular basis for bidirectional communication between the immune and neuroendocrine system. *Physiol Rev* 69. 1-32.

CASTRUCCI AML, HADLEY ME, WILKES BC, ZECHEL C, HRUBY VJ. (1987). Melanin-concentrating hormone exhibits both MSH and MCH activities on individual melanophores. *Life Sci* 40: 1845-1851.

CASTRUCCI AML, LEBL M, HRUBY VJ, MATSUNAGA TO, HADLEY ME. (1989). Melanin-concentrating hormone (MCH). the message sequence. *Life Sci* 45 1141-1148

CHANG C-D, MEIENHOFER J (1978). Solid-phase peptide synthesis using mild base cleavage of N^{α}-fluorenylmethyloxycarbonylamino acids, exemplified by a synthesis of dihydrosomatostatin *Int J Pept Prot Res.* 11: 246-249.

CHOMCZYNSKI P, SACCHI N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction *Anal Biochem.* 162: 156-159.

DE KONING HP, JENKS BG, SCHEENEN WJJM, BALM PHM, ROUBOS EW. (1992). Analysis of autofeedback mechanisms in the secretion of pro-opiomelanocortin-derived peptides by melanotrope cells of *Xenopus laevis Gen Comp Endocrinol.* 87: 394-401

DE BOLD AJ. (1985). Atrial natriuretic factor. a hormone produced by the heart. *Science* 230: 767-770.

DEMSKI LS. (1971). Feeding and aggressive behavior evoked by hypothalamic stimulation in a cichlid fish. *Comp Biochem Physiol A.* 44 684-692.

DEMSKI LS, KNIGGE KM. (1973). The telencephalon and hypothalamus of the bluegill (*Lepomis macrochirus*) evoked feeding, aggressive and reproductive behavior with representative frontal sections *J Comp Neurol.* 143. 1-16.

DEVI L. (1991). Consensus sequence for processing of peptide precursors at monobasic sites *FEBS Lett.* 280: 189-194

DHARMAMBA M. (1979). Corticosteroids and osmoregulation in fishes. *Proc Indian Natl Acad Sci.* 45: 515-525.

DOERR-SCHOTT J. (1976). Immunohistochemical detection by light and electron microscopy of pituitary hormones in cold blooded vertebrates. I. Fishes and amphibians. *Gen Comp Endocrinol.* 28· 487-512.

DROZDZ R, SIEGRIST W, BAKER BI, CHLUBA-DE TAPIA J, EBERLE AN. (1995) Melanin-concentrating hormone binding to mouse melanoma cells in vitro. *FEBS Lett* 359. 199-202.

DROZDZ R, EBERLE AN. (1995) Binding sites for melanin-concentrating hormone (MCH) in brain synaptosomes and membranes from peripheral tissues identified with highly tritiated MCH. *J Receptor Signal Transduction Res.* 15: 487-502.

EBERLE AN. (1988) Melanin-concentrating hormone *In*: The melanotropins Chemistry, physiology and mechanisms of action, pp 321-332. Karger, Basel, Switzerland

EBERLE AN, BAKER BI, KISHIDA M, BAUMANN JB, GIRARD J. (1989). Development of a sensitive solid-phase radioimmunoassay for melanin-concentrating hormone. *Life Sci.* 45: 1149-1154.

EDDY FB. (1981) Effects of stress on osmotic and ionic regulation in fish *In*· Stress and fish, pp 77-102. Pickering AD, ed. Academic Press, London, UK.

EVANS DH. (1979). Osmotic and ionic regulation by freshwater and marine fishes. *In* Environmental physiology of Fishes, pp 93-122. Ali MA, ed. Plenum Press, New York, USA.

FELLMANN D, RISOLD PY, BAHJAOUI M, COMPAGNONE N, BRESSON JL, CLAVEQUIN MC, CARDOT J, GOUGET A, LENYS D, BUGNON C. (1993) Morphofunctional studies on the neurons producing melanin-concentrating hormone. *Ann NY Acad Sci.* 680. 511-516.

FRANCIS K, BAKER BI. (1995). Developmental changes in melanin-concentrating hormone in *Rana temporia. Gen Comp Endocrinol* 98. 157-165.

FRYER JN, LEDERIS K, RIVIER J. (1984) Cortisol inhibits the ACTH-releasing activity of urotensin, CRF and sauvagine observed with superfused goldfish pituitary cells. *Peptides* 5: 925-930.

FUIJI R, MIYASHITA Y. (1976). Receptor mechanisms in fish chromatophores. III. Neurally controlled melanosome aggregation in a siluroid (*Parasilurus asotus*) is strangely mediated by cholinoceptors. *Comp Biochem Physiol C* 55 43-49.

GILHAM ID, BAKER BI. (1985). A black background facilitates the response to stress in teleosts. *J Endocrinol* 105: 99-105.

GORDON-WEEKS PR. (1988). RNA-transport in dendrites. *Trends Neurosci.* 11: 342-343.

GREEN JA, BAKER BI. (1991). The influence of repeated stress on the release of melanin-concentrating hormone in the rainbow trout. *J Endocrinol.* 128 261-266.

GREEN JA, BAKER BI, KAWAUCHI H. (1991) The effect of rearing rainbow trout on black or white backgrounds on their secretion of melanin-concentrating hormone and their sensitivity to stress *J Endocrinol.* 128: 267-274.

GRÖNEVELD, D., HUT M.J., BALM, P.H.M., MARTENS, G.J.M., WENDELAAR BONGA, S.E. (1993) Cloning and sequence analysis of hypothalamus cDNA encoding tilapia melanin- concentrating hormone *Fish Physiol Biochem.* 11: 117-124.

GRÓNEVELD D, ECKHARDT ERM, COENEN AJM, MARTENS GJM, BALM PHM, WENDELAAR BONGA SE. (1995a). Expression of tilapia prepro-melanin-concentrating hormone mRNA in hypothalamic and neurohypophysial cells. *J Mol Endocrinol.* 14. 199-207

GRONEVELD D, BALM PHM, MARTENS GJM, WENDELAAR BONGA SE. (1995b) Differential melanin-concentrating hormone gene expression in two hypothalamic nuclei of the teleost tilapia in response to environmental changes. *J Neuroendocrinol.* (In press)

GRÖNEVELD D, BALM PHM, WENDELAAR BONGA SE. (1995c) Biphasic effect of MCH on α-MSH release from the tilapia (*Oreochromis mossambicus*) pituitary. *Peptides* (In press).

GRONEVELD D, BALM PHM, WENDELAAR BONGA SE (1995d) Identification, cellular localization and *in vitro* release of a novel teleost melanin-concentrating hormone gene related peptide *Neuroendocrinology* (In press).

HADLEY ME, CASTRUCCI AML, HRUBY VJ. (1988) Melanin-concentrating hormone (MCH) mechanisms of action. *In*: Advances in pigment cell research, pp 513-545. Bagnara JY, ed Alan R Liss, New York, USA.

HARRIS RB. (1989). Processing of pro-hormone precursor proteins. *Arch Biochem Biophys.* 275 315-333.

HERVIEU G, NAHON JL. (1995). Pro-melanin concentrating hormone messenger ribonucleic acid and peptides expression in peripheral tissues of the rat. *Neuroendocrinology* 61: 348-364.

HIMICK BA, PETER RE. (1995). Bombesin-like immunoreactivity in the forebrain and pituitary and regulation of anterior pituitary hormone release by bombesin in goldfish. *Neuroendocrinology* 61 365-376.

HOLMES RL, BALL JN. (1974). The hypothalamus and neurohypophysis in teleosts. *In*. The pituitary gland. A comparative account, pp 200-204 Cambridge University Press, Cambridge, UK.

JEZOVA D, BARTANUSZ V, WESTERGEN I, JOHANSON BB, RIVIER J, VALE W, RIVIER C. (1992) Rat melanin-concentrating hormone stimulates adrenocorticotropin secretion. evidence for a site of action in brain regions protected by the blood-brain barrier. *Endocrinology* 130. 1024-1029

KAH O, ANGLADE I, LEPRÊTRE E, DUBOURG P, DE MONBRISON D. (1993) The reproductive brain in fish *Fish Physiol Biochem* 11 85-98

KANDEL ER, SCHWARTZ JH, JESSELL TM. (1991) Principles of neural science Part I-III pp 2-269 Elsevier Science Publishing Co, New York

KASUKAWA H, FUJII R. (1985) Receptor mechanisms in fish chromatophores VII Muscarinic cholinoceptors and alpha adrenoceptors, both mediating pigment aggregation, strangely coexist in Corydoras melanophores *Comp Biochem Physiol C* 88 211-215

KAWAUCHI H, KAWAZOE I, TSUBOKAWA M, KISHIDA M, BAKER BI. (1983) Characterization of melanin-concentrating hormone in chum salmon pituitaries *Nature* 305 321-323

KAWAUCHI H. (1989) Structure and biosynthesis of melanin-concentrating hormone *Life Sci* 45 1133-1140

KAWAZOE I, KAWAUCHI H, HIRANO T, NAITO N. (1987) Structure-activity relationships of melanin-concentrating hormone *Int J Pept Prot Res* 29 714-721

KISHIDA M, BAKER BI, BIRD DJ. (1988) Localization of melanocyte-stimulating hormones in the fish brain *Gen Comp Endocrinol* 71 229-242

KISHIDA M, BAKER BI, EBERLE AN. (1989) The measurement of melanin-concentrating hormone in trout blood *Gen Comp Endocrinol* 74 221-229

KNOLLEMA S, BROWN ER, VALE W, SAWCHENKO PE. (1992) Novel hypothalamic and preoptic sites of prepro-melanin-concentrating hormone messenger ribonucleic acid and peptide expression in lactating rats *J Neuroendocrinol* 4 709-717

KUMAZAWA T, FUJII R. (1984) Concurrent releases of norepinephrine and purines by potassium from adrenergic melanosome-aggregating nerve in Tilapia *Comp Biochem Physiol C* 78 263-266

LAMERS AE, BALM PHM, HAENEN HEMG, JENKS BG, WENDELAAR BONGA SE. (1991) Regulation of differential release of α-MSH forms from the pituitary of a teleost fish, *Oreochromis mossambicus J Endocrinol* 129 179-187

LAMERS AE, FLIK G, ATSMA W, WENDELAAR BONGA SE. (1992) A role for di-acetyl α-melanocyte stimulating hormone in the control of cortisol release in the teleost *Oreochromis mossambicus J Endocrinol* 135 285-292

LAMERS AE, FLIK G, WENDELAAR BONGA SE. (1994) A specific role for TRH in release of diacetyl α-MSH in tilapia stressed by acid water *Am J Physiol* 267 R1302-R1308

LAMERS AE. (1994) α-MSH release in tilapia Why and How *Ph D Thesis* University of Nijmegen, Nijmegen, The Netherlands

MARTENS GJM, JENKS BJ, VAN OVERBEEKE AP. (1981) Nα-acetylation is linked to α-MSH release from pars intermedia of the amphibian pituitary gland *Nature* 294 558-560

MATSUNAGA TO, HRUBY VJ, LEBL M, CASTRUCCI AML, HADLEY ME. (1989) Melanin-concentrating hormone (MCH) structure-function aspects of its melanocyte stimulating hormone-like (MSH-like) activity *Peptides* 10 773-778

MAULE AG, SCHRECK CB, KAATTARI SL. (1987) Changes in the immune system of coho salmon (*Oncorhynchus kisutch*) during parr-to-smolt transformation and after implantation of cortisol *Can J Fish Aquatic Sci* 44 161-166

MERRIFIELD RB. (1963). Solid phase peptide synthesis. I. The synthesis of a tetrapeptide. *J Am Chem Soc.* 85: 2149-2154.

MILLER CL, HRUBY VJ, MATSUNAGA TO, BICKFORD PC. (1993). Alpha-MSH and MCH are functional antagonists in a CNS auditory gating paradigm. *Peptides* 14: 431-440.

MINTH CD, QUI H, AKIL H, WATSON SJ, DIXON JE. (1989) Two precursors of melanin-concentrating hormone: DNA sequence analysis and *in situ* and immunochemical localization *Proc Natl Acad Sci USA.* 86: 4292-4296.

NAHON JL, PRESSE F, BITTENCOURT JC, SAWCHENKO PE, VALE W. (1989) The rat melanin-concentrating hormone messenger ribonucleic acid encodes multiple putative neuropeptides in the dorsolateral hypothalamus. *Endocrinology* 125: 2056-2065

NAHON JL, PRESSE F, SCHOEPFER R, VALE W. (1991) Identification of a single melanin-concentrating hormone messenger ribonucleic acid in coho salmon: structural relatedness with 7SL ribonucleic acid *J Neuroendocrinol* 3. 173-183.

NAHON JL, PRESSE F, BRETON C, HERVIEU G, SCHORPP M. (1993) Structure and regulation of the melanin-concentrating hormone gene *Ann NY Acad Sci.* 680. 111-129.

NAITO N, NAKAI Y, KAWAUCHI H, HAYASHI Y. (1985). Immunocytochemical identification of melanin-concentrating hormone in the brain and pituitary gland of the teleost fishes *Oncorhyncus keta* and *Salmo gairdneri Cell Tissue Res.* 242 41-48.

NAVARRA P, TSAGARAKIS S, COY DH, REES LH, BESSER GM, GROSSMAN AB. (1990). Rat melanin concentrating hormone does not modify the release of CRH-41 from rat hypothalamus or ACTH from the anterior pituitary *in vitro. J Endocrinol.* 127 R1-R4.

NORRIS DO. (1980) Vertebrate endocrinology. Lea & Febiger, Philadelphia, USA.

OHNO S, WOLF U, ATKIN NB. (1968). Evolution from fish to mammals by gene duplication. *Hereditas.* 59: 169-187.

OKAWARA Y, MORLEY SD, BURZIO LO, ZWIERS H, LEDERIS K, RICHTER D. (1988). Cloning and sequence analysis of cDNA for corticotropin-releasing factor precursor from the teleost fish *Catostomus commersoni. Proc Natl Acad Sci USA.* 85. 8439-8443.

OLIVEREAU M. (1967) Observations sur l'hypophyse de l'anguille femelle en particulier lors de la maturation sexuelle. *Z Zellforsch Mikrosk Anat.* 80: 286-306.

ONO M, WADA C, OIKAWA I, KAWAZOE I, KAWAUCHI H. (1988). Structures of two kinds of mRNA encoding the chum salmon melanin-concentrating hormone *Gene* 71 433-438.

PARKES D, VALE W. (1992). Secretion of melanin-concentrating hormone and neuropeptide-EI from cultured rat hypothalamic cells. *Endocrinology* 131 1826-1831

PARKES D, VALE W. (1993). Contrasting actions of melanin-concentrating hormone (MCH) and neuropeptide-E-I (NEI) on posterior pituitary function. *Ann NY Acad Sci.* 680 588-591

PERKS AM. (1987). The neurohypophysis. the place of comparative studies in biology and medicine. *In*: Vertebrate endocrinology: fundamentals and biomedical implications, Vol. 2. Pang PKT, Schreibman MP, eds. Academic Press, Orlando, USA.

PERLMAN D, HALVORSON HO. (1983) A putative signal peptidase recognition site and sequence in eukaryotic and prokaryotic signal peptides. *J Mol Biol.* 167: 391-409.

PETER RE, FRYER JN. (1983). Endocrine functions of the hypothalamus of actinopterygians. *In*: Fish neurobiology, Vol. 2, pp 165-201 Davis RE, Northcutt RG, eds. The University of Michigan Press, Ann Harbor, USA.

PETER RE, GILL VE. (1990). A stereotaxic atlas and technique for forebrain nuclei of the goldfish, *Carassius auratus J Comp Neurol.* 159: 69-102.

PETER RE, YU K-L, MARCHANT TA, ROSENBLUM PM. (1990). Direct neural regulation of the teleost neurohypophysis. *J Exp Zool Suppl* 4. 84-89

PICKERING AD, POTTINGER TG, SUMPTER JD. (1987). On the use of dexamethasone to block the pituitary-interrenal axis in the brown trout, *Salmo trutta* L. *Gen Comp Endocrinol.* 65. 346-353.

PICKFORD GE, ATZ J. (1957). The physiology of the pituitary gland of fishes. Zoological Society. New York, USA.

POWEL KA, BAKER BI. (1988). Structural studies of nerve terminals containing melanin-concentrating hormone in the eel, *Anguilla anguilla. Cell Tissue Res.* 251 433-439.

PRESSE F, NAHON J-L, FISCHER WH, VALE W. (1990). Structure of the human melanin-concentrating hormone mRNA *Mol Endocrinol.* 4: 632-637.

PRESSE F, NAHON JL. (1993). Differential regulation of melanin-concentrating hormone gene expression in distinct hypothalamic areas under osmotic stimulation in rat *Neuroscience* 55. 709-720.

PRESSE F, HERVIEU G, IMAKI T, SAWCHENKO PE, VALE W, NAHON JL. (1992). Rat melanin-concentrating hormone messenger ribonucleic acid expression. marked changes during development and after stress and glucocorticoid stimuli. *Endocrinology* 131: 1241-1250.

PROUDFOOT NJ, BROWNLEE GG. (1976) 3' non-coding region sequences in eukaryotic messenger RNA. *Nature* 263 211-214.

RODRIGUES KT, SUMPTER JP. (1984). Effects of background adaptation on the pituitary and plasma concentrations of some pro-opiomelanocortin-related peptides in the rainbow trout (*Salmo gairdneri*) *J Endocrinol.* 101: 277-284.

ROITT I, BROSTOFF J, MALE D. (1989). Immunology Gower Medical Publishing, London, UK.

ROSENBLUM PM, GOOS JTH, PETER RE.: Regional distribution and *in vitro* secretion of salmon and chicken-II gonadotropin-releasing hormones from the brain and pituitary of juvenile and adult goldfish, *Carassius auratus. Gen Comp Endocrinol.* 93: 369-379

RUDMAN D, HOLLINS BM, KUTNER MH, MOFFITT SD, LYNN MJ. (1983). Three types of α-melanocyte-stimulating hormone: bioactivities and half-lives. *Am J Physiol.* 245 E47-E54

SALM AK, HATTON GI, NILAVER G. (1982). Immunoreactive glial fibrillary acidic protein in pituicytes of the rat neurohypophysis. *Brain Res* 236 471-476

SAMBROOK J, FRITSCH EF, MANIATIS T. (1989). Molecular cloning: a laboratory manual (2nd ed.) Cold Spring Harbor University Press, Cold Spring Harbor, New York

SANGER, F., NICKLEN, S. AND COULSON, A.R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA.* 74· 5463-5467.

SCHÄFER MHK, DAY R, ORTEGA MR, AKIL H, WATSON SJ. (1990). Proenkephalin messenger RNA is expressed both in the rat anterior and posterior pituitary *Neuroendocrinology* 51: 444-448.

SCHELLER, R.H., KALDANY, R.R., KREINER, T., MAHON, A.C., NAMBU, J.R., SCHEFER, M. AND TAUSSIG, R. (1984). Neuropeptides: mediators of behaviour in *Aplysia Science* 225: 1300-1308.

SCHRECK CB, BRADFORD C, FITZPATRIC MS, PATIÑO R. (1989). Regulation of the interrenal of fishes: non-classical control mechanisms. *Fish Physiol Biochem.* 7: 259-265.

SCHWARTZ TW. (1986). The processing of peptide precursors. 'Proline directed arginnyl cleavage' and other monobasic processing mechanisms. *FEBS Lett* 200: 1-10

SHERBROOKE WC, HADLEY ME, CASTRUCCI AML. (1988). Melanotropic peptides and receptors an evolutionary perspective in vertebrate physiological color change *In* The melanotropic peptides, Vol II, pp 175-190. Hadley ME, ed. CRC press, Boca Raton, USA.

SMRIGA M, BAKOS P, JEZOVÁ D. (1994) Influence of salmon melanin-concentrating hormone on vasopressin analogue (dDAVP) activity and sodium transport in frog skin *Gen Physiol Biophys.* 13: 413-424.

STEWART JM, YOUNG JD. (1984). The chemistry of solid phase peptide synthesis *In*: Solid phase peptide synthesis, second edition, pp 1-52. Pierce Chemical Company, Rockford, USA.

TAKAHASHI K, SUZUKI H, TOTSUNE K, MURAKAMI O, SATOH F, SONE M, SASANO H, MOURI T, SHIBAHARA S. (1995) Melanin-concentrating hormone in human and rat. *Neuroendocrinology* 61. 493-498

TAKEI Y, TAKAHASHI A, WATANABE TX, NAKAJIMA K, SAKAKIBARA S. (1989) Amino acid sequence and relative biological activity of eel atrial natriuretic peptide *Biochem Biophys Res Comm.* 164 537-543.

TALLARIDA RJ, MURRAY RB. (1986) Manual of pharmacological calculations with computer programs, second edition. Springer-Verlag, New York, USA.

TENSEN CP, COENEN T, VAN HERP F. (1991). Detection of mRNA encoding crustacean hyperglycemic hormone (CHH) in the eyestalk of the cray fish *Orconectus limosus* using non-radioactive in situ hybridization. *Neurosci Lett.* 124. 178-182.

THEODOSIS DT, POULAIN DA. (1992). Neuronal-glial and synaptic plasticity of the adult oxytocinergic system *Ann NY Acad Sci* 652. 303-325.

TRAN TN, FRYER JN, BENNETT HPJ, TONON MC, VAUDRY H. (1989) TRH stimulates the release of POMC-derived peptides from goldfish melanotropes *Peptides* 10 835-841

TWEEDLE CD, HATTON GI. (1987). Morphological adaptability at neurosecretory axonal endings on the neurovascular contact zone of the rat neurohypophysis. *Neuroscience* 20 241-246.

VALLARINO M, ANDERSEN AC, DELBENDE C, OTTONELLO I, EBERLE AN, VAUDRY H. (1989) Melanin-concentrating hormone (MCH) immunoreactivity in the brain and pituitary of the dogfish *Scyliorhinus canicula*. Colocalisation with alpha-melanocyte-stimulating hormone (α-MSH) in hypothalamic neurons *Peptides* 10: 375-382.

VAN EYS GJJM. (1981). Physiological and biochemical aspects of the teleostean pars intermedia *Ph D Thesis*. University of Nijmegen, Nijmegen, The Netherlands

VAN TOL HHM, BURBACH JPH. (1989) Quantitation of vasopressin and oxitocin mRNA levels in the brain. *Meth Enzymol.* 168. 398-413.

VAUGHAN JM, FISCHER WH, HOEGER C, RIVIER J, VALE W. (1989). Characterization of melanin-concentrating hormone from rat hypothalamus. *Endocrinology* 125: 1660-1665.

VERBOST PM, SCHOENMAKERS THJM, FLIK G, WENDELAAR BONGA SE. (1994). Kinetics of ATP- and Na^+-gradient driven Ca^{2+} transport in basolateral membranes from gills of freshwater- and seawater-adapted tilapia. *J Exp Biol* 186: 95-108.

VON HEIJNE G. (1986). A new method for predicting signal sequence cleavage sites *Nucleic Acids Res.* 14. 4683-4690

WARING H. (1963). Colour change mechanisms of cold-blooded vertebrates Academic Press, New York. USA

WENDELAAR BONGA SE. (1970). Ultra-structure and histochemistry of neurosecretory cells and neurohaemal areas in the pond snail *Lymnea stagnalis* (L.). *Z Zellforsch mikros Anat.* 108: 190-224

WENDELAAR BONGA SE. (1993). Endocrinology. *In*: The physiology of fishes, pp 469-502. Evans DH, ed. CRC Press, Boca Raton, USA.

WENDELAAR BONGA SE, BALM PHM. (1989). Endocrine responses to acid stress in fish. *In* Acid toxicity and aquatic animals, pp 243-263. Morris R, Taylor EW, Brown DJA, Brown JA, eds. Cambridge university Press, Cambridge, UK.

WILKES BC, HRUBY VJ, CASTRUCCI AML, SHERBROOKE WC, HADLEY ME. (1984) Synthesis of a cyclic melanotropic peptide exhibiting both melanin-concentrating and -dispersing activities. *Science* 224: 1111-1113.

YU KL, ROSENBLUM PM, PETER RE. (1991). *In vitro* release of gonadotropin-releasing hormone from the brain preoptic-anterior hypothalamic region and pituitary of female goldfish. *Gen Comp Endocrinol.* 81: 256-267

ZAMIR N, SKOFITSCH G, BANNON MJ, JACOBOWITZ DM. (1986). Melanin-concentrating hormone: unique peptide neuronal system in the rat brain and pituitary gland *Proc Natl Acad Sci USA.* 83· 1528-1531.

Samenvatting voor niet ingewijden (Summary in Dutch)

Melanine-concentrerend hormoon (MCH) is een hormoon dat het eerst is aangetoond in vissen, maar recentelijk werd duidelijk dat zoogdieren en mensen ook MCH aanmaken Het is al lang bekend dat MCH in vissen belangrijk is bij de aanpassing van de huidkleur aan een lichte omgevingskleur Veel vissoorten krijgen een lichtere huidkleur als je ze in een wit aquarium laat zwemmen Die lichte kleur ontstaat doordat het pigment (melanine) in de pigmentcellen van de schubben en de huid daaronder vanuit een stervorm wordt geconcentreerd tot een klein puntje MCH zorgt voor deze melanine concentratie Zoogdieren en mensen passen hun huidkleur niet aan aan hun omgeving, dus bij deze soorten moet MCH een andere functie hebben dan regulatie van melanine concentratie Een interessante vraag die daardoor ontstond was of MCH in vissen dan ook nog andere functies heeft Het oplossen van deze vraag was de belangrijkste doelstelling van mijn promotieonderzoek

Een goede manier om te zoeken naar een functie van een hormoon is te kijken onder welke omstandigheden de aanmaak en/of de afgifte van dat hormoon wordt verhoogd In een situatie waarin een hormoon belangrijk is zal daarvan meer nodig zijn en zal er ook meer van worden gemaakt en afgegeven Voordat met het onderzoek van dit proefschrift een aanvang werd genomen, was het bijvoorbeeld al bekend dat er in het bloed van forellen die in een wit aquarium gehouden werden meer MCH aanwezig is dan in het bloed van vissen uit een zwart aquarium Dus op een witte achtergrond wordt meer MCH afgegeven dan op een zwarte achtergrond

Bij de zoektocht naar een nieuwe functie van MCH in vissen heb ik de tilapia, een Afrikaanse natalbaars (ook wel *Oreochromis mossambicus* genoemd) gekozen als proefdier Uit voorafgaand onderzoek in onze vakgroep was namelijk gebleken dat deze vis zich erg goed kan aanpassen aan verschillende omstandigheden Tilapia's passen hun huidkleur bijvoorbeeld goed aan aan hun omgeving In een wit aquarium zijn ze heel licht, terwijl ze in een zwart aquarium zo donker zijn dat je ze bijna niet kunt zien Een ander voorbeeld is de goede aanpassing van deze vis aan veranderingen in het zout- en zuurgehalte van het water

Je kunt de tilapia namelijk probleemloos overbrengen van zoetwater naar zeewater of naar behoorlijk verzuurd water Een tweede reden voor het gebruik van de tilapia voor mijn onderzoek was de beschikbaarheid van een groot aantal analysetechnieken op ons laboratorium om te bepalen of deze vis zich kan aanpassen aan de veranderingen of dat hij dit niet meer goed kan en daardoor gestresst raakt Van een aantal van deze analysetechnieken heb ik gebruik gemaakt om te controleren of de tilapia zich daadwerkelijk aanpaste aan de toegediende behandelingen

Voordat ik me aan de hoofdvraagstelling van mijn onderzoek kon gaan wijden moest eerst enig voorwerk worden verricht Om aanmaak van MCH in tilapia onder verschillende omstandigheden te kunnen bestuderen moest eerst een methode worden ontwikkeld om nieuw aangemaakt MCH te *herkennen* Met die methode werd de precieze *locatie* van de cellen die dit MCH produceren bepaald Daarna werd een methode opgezet om de *hoeveelheid* nieuw aangemaakt MCH in deze cellen te bepalen MCH bleek in tilapia aangemaakt te worden in drie verschillende celgroepen Twee groepen met MCH-cellen zijn gelocaliseerd in de hersenen, in gebieden die respectievelijk nucleus lateralis tuberis (afgekort NLT) en nucleus recessus lateralis (NRL) worden genoemd De aanwezigheid van MCH cellen in deze hersengebieden was een bevestiging van wat in een aantal andere vissen al eerder was gevonden Nieuw was de vondst van een derde (kleine) groep van MCH-cellen in de hypofyse (hersenaanhangsel) De hypofyse is een klier waarin verschillende hormonen worden gemaakt en van waaruit ook hormonen die in de hersenen worden gemaakt (zoals MCH van vissen) worden afgegeven aan het bloed De hoeveelheid nieuw aangemaakt MCH in de hypofyse was moeilijk te bepalen, omdat er maar heel weinig MCH cellen aanwezig zijn in deze klier Ik heb me daarom bij het verdere onderzoek gericht op de MCH-cellen in de hersenen Omdat ik me afvroeg of de aanmaak van MCH in twee verschillende celgroepen in de vissehersenen een functionele betekenis heeft, heb ik geprobeerd functies te vinden voor beide celgroepen Hiertoe heb ik onder verschillende omstandigheden de hoeveelheid aangemaakt MCH in deze celgroepen afzonderlijk bepaald met een speciaal daarvoor opgezette methode Omdat alleen in de NLT meer MCH bleek te worden aangemaakt als de vissen in een wit aquarium waren gehouden (Fig S 1), heb ik geconcludeerd dat deze celgroep (en niet de MCH-cellen van de NRL) verantwoordelijk is voor de bekende functie van MCH in de aanpassing van de huidkleur aan de omgeving De hoeveelheid aangemaakt MCH in de NLT was ook vergroot wanneer tilapia's ernstig gestresst waren door ze in zeer verzuurd water te laten zwemmen, maar niet wanneer de vissen waren blootgesteld aan mildere verzuring, waaraan ze zich goed konden aanpassen Dit is dus een duidelijke aanwijzing dat deze MCH-cellen ook een functie hebben tijdens ernstige stress

De MCH cellen van de NRL bleken gevoelig te zijn voor een behandeling die 'herhaalde verstoring' werd genoemd De herhaalde verstoring bestond uit drie onderdelen die dagelijks,

elk gedurende tien minuten werden herhaald, namelijk het uitschakelen van de verlichting, het uitzetten van zuurstoftoevoer en het plaatsen van de vissen samen in een klein netje Door deze herhaalde verstoring werd in de NRL meer MCH aangemaakt dan onder controle omstandigheden (Fig S 1) De MCH-cellen van deze hersenregio hebben dus waarschijnlijk een functie bij de regulatie van de fysiologische aanpassing aan 'verstoring'
Samengevat kan worden gesteld dat uit mijn onderzoek een nieuwe mogelijke functie van MCH naar voren is gekomen, namelijk betrokkenheid bij de regulatie van processen tijdens 'stress' en/of 'verstoring' MCH zou ook deze functie kunnen hebben bij zoogdieren In de zeer recente literatuur zijn daar ook al aanwijzingen voor

Een tweede vraagstelling van mijn onderzoek betrof de vraag wat de doelwitorganen of -cellen zijn van de MCH-cellen tijdens stress en bij de regulatie van huidkleuraanpassing Daarom heb ik onderzocht of de MCH-cellen aangrijpingspunten hebben in de hypofyse In deze klier worden namelijk en tweetal hormonen - adrenocorticotroop hormoon (ACTH) en melanocyten stimulerend hormoon (MSH) - geproduceerd, waarvan bekend is dat ze een rol spelen bij de regulatie van de stress-respons in vissen MSH speelt bovendien, net als MCH, ook een rol bij de aanpassing van de huidkleur aan de achtergrond door ervoor te zorgen dat de vissen op een donkere achtergrond donker worden

Voordat ik verder inga op de effecten van MCH-cellen op hypofyseniveau, moet worden vermeld dat MCH-cellen niet alleen MCH aanmaken Bij het ontwikkelen van de methode om nieuw aangemaakt MCH te herkennen, bleek dat tegelijk met MCH ook nog een andere factor, het zogenaamde MCH-gen-gerelateerde peptide (of korter Mgrp), kan worden geproduceerd De aanmaak en afgifte van Mgrp bij vissen is in dit proefschrift voor het eerst beschreven

Om de aangrijpingspunten van de MCH-cellen in de hypofyse te bepalen zijn de effecten van MCH en Mgrp op de afgifte van MSH en ACTH bestudeerd Deze uit MCH-cellen afkomstige produkten bleken verschillende effecten te hebben op de MSH afgifte Kleine hoeveelheden MCH bleken de MSH afgifte te remmen, terwijl grote hoeveelheden de MSH afgifte juist stimuleren (Fig S 1) Omdat het op een lichte achtergrond van belang is dat er veel MCH (veroorzaakt lichter worden van de huid) en weinig MSH (veroorzaakt donkerkleuring) aanwezig is, veronderstel ik dat het remmende effect van MCH op de MSH afgifte waarschijnlijk optreedt tijdens huidkleuraanpassing Het stimulerende effect is mogelijk van belang tijdens de stress-respons, maar daarop zal ik verderop terugkomen Mgrp bleek, in tegenstelling tot MCH, geen effect te hebben op de MSH afgifte en ook niet op de kleur van de huid

Bij de regulatie van de stress-respons speelt naast MSH ACTH een belangrijke rol Omdat MCH-cellen gevoelig bleken te zijn voor stress en Mgrp geen effect bleek te hebben op de MSH afgifte, heb ik onderzocht of dit Mgrp invloed heeft op de ACTH afgifte Mgrp

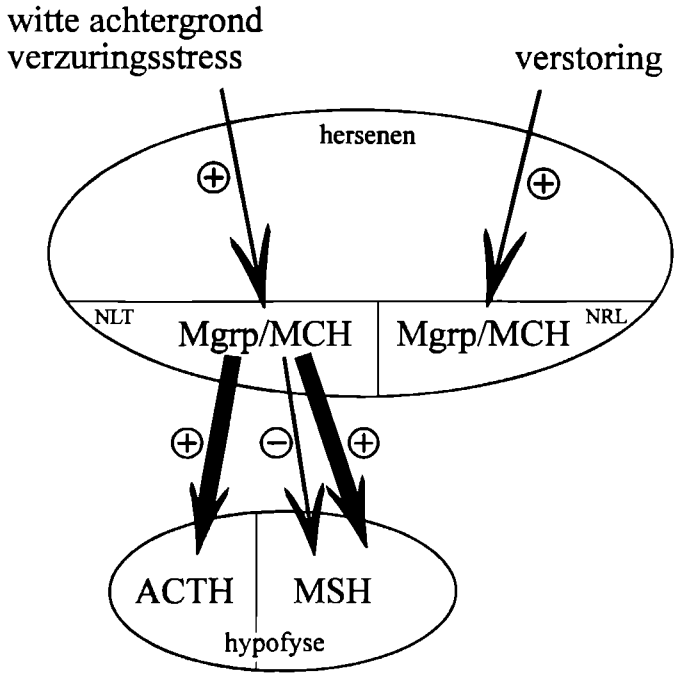
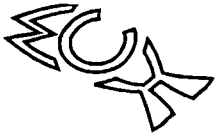


Fig. S.1 Schematische weergave van het MCH-systeem van tilapia MCH en Mgrp worden in de MCH-cellen van de NLT en NRL aangemaakt De aanmaak van MCH en Mgrp in de NLT is verhoogd als de vissen op een witte achtergrond worden gezet en als ze worden blootgesteld aan ernstige verzuringsstress De aanmaak van deze produkten in de MCH-cellen van de NRL is verhoogd als de dieren worden verstoord Het MCH uit de NLT kan de MSH afgifte uit de hypofyse remmen (bij toediening van weinig MCH, dunne pijl) en stimuleren (bij veel MCH, dikke pijl), terwijl Mgrp de afgifte van ACTH kan stimuleren (bij toediening van veel Mgrp)

bleek de ACTH afgifte te stimuleren bij dezelfde grote hoeveelheden als waarbij MCH de MSH afgifte stimuleert (Fig S 1) Op grond van de bovenstaande resultaten veronderstel ik dat de stimulerende effecten van MCH op de MSH afgifte en van Mgrp op de ACTH afgifte in verband staan met de activatie van MCH-cellen bij verzuringsstress

Samengevat is in dit proefschrift een 'nieuwe' functie van MCH-cellen in vissen beschreven, namelijk in de regulatie van processen tijdens stress en verstoring Daarnaast is aangetoond dat de produkten van de MCH-cellen, MCH en Mgrp, aangrijpen op respectievelijk de MSH en de ACTH cellen in de hypofyse, die een rol spelen bij de huidkleuraanpassing aan de omgeving (MSH) en in de stress-respons (MSH en ACTH)



Dankwoord
(Acknowledgements)

In Nijmegen promoveer je niet alleen Dat is wat ik ervaren heb in de vijf jaren dat ik mijn promotieonderzoek heb verricht op de afdeling experimentele dierkunde Zoals gebruikelijk werd het werk dat in dit proefschrift is beschreven uitgevoerd in samenwerking met velen Daarom wil ik op deze plaats graag iedereen bedanken die op enigerlei wijze heeft bijgedragen aan de totstandkoming van dit boekje

Bij Sjoerd Wendelaar Bonga en Paul Balm, mijn promotor en co-promotor kon ik altijd terecht als het om de fysiologie van vissen ging Gerard Martens wil ik graag bedanken voor de mogelijkheid die hij mij heeft geboden om te "integreren" in zijn moleculair biologische werkgroep Bij een belangrijk deel van mijn onderzoek heb ik kunnen profiteren van jouw know-how In de slotfase bij het opschrijven van mijn resultaten waren de discussies met Gert Flik een welkome aanvulling op de meningen van de andere "bazen" Jouw opbouwende kritiek en enthousiasme waren voor mij zeer motiverend bij de laatste loodjes

De afgelopen jaren heb ik de meeste tijd doorgebracht op wat nu "de derde" heet Anneke, Cees, Doug, Eric, François, Joost, Karel, Maarten, Martin en Tony, ik vond het leuk om met jullie samen te werken en ook de gezamenlijke buitenwerkse activiteiten vond ik altijd een succes Een speciaal bedankje verdient Dominique Ik kon op "jouw lab" terecht toen de anderen me boven hadden "verdreven ' Jouw belangstelling voor mijn werk en voor zaken die daar niets mee te maken hebben heb ik zeer gewaardeerd

Later verplaatsten mijn activiteiten zich meer naar de tweede en soms naar de vierde verdieping Hier zorgden Anne, Angelique, Declan, Elze, Frank, Leon, Liesbeth, Peter, Pieter, Ron, Stanny, Sylvia, Tom en Xander voor de nodige ondersteuning en lol tijdens het werk Anne, jouw inbreng op het lab en op reis was zo groot dat je wel paranimf moest worden Fijn dat je speciaal daarvoor uit Nice kunt komen en sorry dat jouw schatjes nu hun bazin moeten missen op dierendag

En dan mijn kamergenoten van het laatste uur, Marcel, Peter en Theo Ik vind het geweldig dat ik jullie computer tot de "mijne' heb mogen maken en dat jullie me nog hielpen ook Daarnaast wil ik jullie bedanken voor jullie belangstelling voor alles wat met wetenschap en andere bezigheden te maken had Peter, succes in Sheffield

Tijdens mijn OIO-schap heb ik drie studenten mogen begeleiden Eric, Mark en Ton, ik hoop dat jullie er net zoveel plezier aan hebben beleefd als ik. Een deel van jullie resultaten is opgenomen in dit boekje

Promoveren lukt niet met alleen wetenschappelijke inspanning Regelmatige ontspanning is onontbeerlijk. Daarom wil ik de atleten van het Richard Riley Running Team bedanken voor alle ontspannende, maar vaak ook (lichamelijk) inspannende ondernemingen Mijn waardering hiervoor blijkt wel uit het feit dat Richard nu paranimf is

Tenslotte wil ik mijn ouders bedanken voor hun vertrouwen in mij Jullie stimulerende en gelukkig op z'n tijd ook relativerende invloed droeg er in belangrijke mate toe bij dat mijn wetenschappelijke opleiding met dit proefschrift kon worden afgerond

Curriculum vitae

De schrijfster van dit proefschrift werd geboren op 11 juli 1966 in Enschede Nadat de basis van haar scholing was gelegd op de Openbare Cornelis Jetsesschool te Enschede, behaalde zij in 1984 het Atheneum B diploma aan de Gemeentelijke Scholengemeenschap Zuid in dezelfde stad In het najaar van 1984 begon zij haar studie Biologie aan de Katholieke Universiteit Nijmegen Na de propadeuse in juni 1985, werd het doctoraal examen in de Toegepaste Richting behaald in juni 1990, met als hoofdvak Moleculaire Biologie (bij Prof Dr J G Schoenmakers en Prof Dr G J M Martens) en als bijvakken Biochemie (bij Dr R J Hamer, TNO IGMB in Wageningen), Moleculaire Plantenfysiologie (Prof Dr G Wullems en Dr A F Croes) en Immunologie (Prof Dr J H Berden en Dr R M Termaat) Van juli 1990 tot juli 1994 was zij werkzaam als onderzoeker in opleiding bij de vakgroep Experimentele Dierkunde aan de Katholieke Universiteit van Nijmegen, in dienst van NWO, binnen het NWO onderzoeksthema "Neuropeptiden en gedrag" In deze periode verrichtte zij het onderzoek dat in dit proefschrift is beschreven Daarnaast behaalde zij de artikel 9 bevoegdheid Proefdierkunde en nam zij deel aan de cursus Management voor AIO's en de cursus Neurobiologie van de Onderzoeksschool Pathofysiologie van het zenuwstelsel, die zij beiden met goed gevolg afsloot Tevens werd een bijdrage geleverd aan het biologie-onderwijs in de vorm van begeleiding van doctoraal-studenten

Naast deelname aan jaarlijkse symposia en bijeenkomsten binnen Nederland, presenteerde zij haar onderzoeksresultaten tijdens het '11th International Symposium on Neurosecretion', in juni 1991 (Amsterdam), het '2nd International Symposium on Fish Endocrinology" in juni 1992 (Saint-Malo, Frankrijk) en de "17th Conference of European Comparative Endocrinologists" in september 1994 (Córdoba, Spanje)


MILIEUKEURMERK